PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/53, 15/82, C12Q 1/26, 1/02, A01H 5/00

A1

(11) International Publication Number: WO 97/49816

(43) International Publication Date: 31 December 1997 (31.12.97)

(21) International Application Number: PCT/US97/11295

(22) International Filing Date: 26 June 1997 (26.06.97)

(30) Priority Data:
60/021,364 27 June 1996 (27.06.96) US

(71) Applicant *(for all designated States except US)*: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MAXWELL, Carl, Arthur [US/US]; 35 Mary Anita Court, Elkton, MD 21921 (US). SCOLNIK, Pablo, Ariel [US/US]; 120 Spottswood Lane, Kennett Square, PA 19348 (US). WITTENBACH, Vernon, Arie [US/US]; 609 Greenbank Road, Wilmington, DE 19808 (US). GUTTERIDGE, Steven [GB/US]; 4 Austin Road, Wilmington, DE 19810 (US).

(74) Agent: FLOYD, Linda, A.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: PLANT GENE FOR *P*-HYDROXYPHENYLPYRUVATE DIOXYGENASE

```
1     CAAGAAACGNGTCGNCGACGTGCTCAGCGATGATCAGATCAAGGAGTGTGAGGAATTAGG
61    GATTCTTNTAGACAGAGATGATCAAGGGACGTTNCTTCAAATCTNCACAAAACCACTAGG
121   TGACAGGCCGACGNTATTTATAGAGATAATCCAGAGNGTAGGATGCATGATGAAAGATGT
181   GGAAGGGANGGCTTACCAGAGTGGAGNATNTNGTGGTTTTGGCAAAGGCAATT
```

(57) Abstract

The invention relates to the isolation and modification of nucleic acid sequences encoding *p*-hydroxyphenylpyruvate dioxygenase enzyme from plants. These nucleic acid sequences were used to establish methods of identification of new herbicidal compounds that inhibit the activity of this enzyme, and to prepare new crop plants that are tolerant to the herbicidal action of inhibitors of this enzyme. Chimeric genes comprising nucleic acid fragments containing all or part of the nucleic acid sequences encoding *p*-hydroxyphenylpyruvate dioxygenase may be used to produce active plant *p*-hydroxyphenylpyruvate dioxygenase enzyme in microorganisms, and to cause the production of modified forms of the enzyme in plants that may render such plants tolerant to inhibitors of the enzyme.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

TITLE

PLANT GENE FOR *P*-HYDROXYPHENYLPYRUVATE DIOXYGENASE

FIELD OF THE INVENTION

This invention relates to the isolation and modification of nucleic acid encoding *p*-hydroxyphenylpyruvate dioxygenase enzyme from plants. These nucleic acid sequences were used to establish methods of identification of new herbicidal compounds that inhibit the activity of this enzyme, and to prepare new crop plants that are tolerant to the herbicidal action of inhibitors this enzyme. Chimeric genes comprising nucleic acid fragments containing all or part of the nucleic acid sequences encoding *p*-hydroxyphenylpyruvate dioxygenase may be used to produce active plant *p*-hydroxyphenylpyruvate dioxygenase enzyme in microorganisms, and to cause the production of modified forms of the enzyme in plants that may render such plants tolerant to inhibitors of the enzyme.

BACKGROUND OF THE INVENTION

Bleaching herbicides affect plant chloroplasts by decreasing their chlorophyll and carotenoid content. Several bleaching herbicides are known to inhibit the enzyme phytoene desaturase, resulting in the accumulation of phytoene in treated plants. However, compounds of the benzoyl cyclohexane-1,3-dione type cause the accumulation of phytoene in plants but are not inhibitors of phytoene desaturase *in vitro* (Sandmann, G., et al. (1990) *Pestic. Sci.* 30:353-355). Subsequent work revealed that these compounds are effective inhibitors of *p*-hydroxyphenylpyruvate dioxygenase (*p*-hydroxyphenylpyruvate:oxygen oxidoreductase EC 1.13.11.27), a key enzyme in the biosynthesis of plastoquinones and tocopherols (Schulz, A., et al. (1993) *FEBS Lett.* 318:162-166). Based on the observation that phytoene desaturase requires a quinone as an electron acceptor, these authors postulated that by inhibiting *p*-hydroxyphenylpyruvate dioxygenase, these herbicides act indirectly on phytoene desaturase by blocking the biosynthesis of quinones.

The proposal that *p*-hydroxyphenylpyruvate dioxygenase is essential for carotenoid biosynthesis has received support from genetic studies in the plant model system *Arabidopsis thaliana*. Mutations in the *pds1* and *pds2* genetic loci result in mutant plants that accumulate phytoene. However, genetic mapping of these mutant genes indicates that they do not correspond to the gene encoding the enzyme phytoene desaturase. The *pds1* mutation can be rescued by homogentisic acid, the substrate of *p*-hydroxyphenylpyruvate dioxygenase. Therefore, this mutation corresponds to a defect in the activity of *p*-hydroxyphenylpyruvate dioxygenase (Norris, S. R., et al. (1995) *Plant Cell* 7:2139-2149).

In light of these disclosures, *p*-hydroxyphenylpyruvate dioxygenase is a promising new target for new herbicidal compounds. Research aimed at discovering new herbicides based on this mode of action would be greatly facilitated by the isolation of the plant gene encoding this enzyme and by the functional expression of this gene in transgenic organisms. For example, active enzyme produced in recombinant microorganisms could be used to establish screening methods for the identification of novel active compounds and to obtain structural and mechanistic information useful to guide further chemical synthesis. Furthermore, isolation of this gene would facilitate research aimed at generating mutant, herbicide-tolerant versions of the enzyme that may confer herbicide resistance to transgenic plants.

A partial sequence of an *Arabidopsis thaliana* cDNA with homology to corresponding mammalian sequences encoding *p*-hydroxyphenylpyruvate dioxygenase has been identified (GenBank Accession No. T20952), but this truncated sequence is insufficient to identify an active plant *p*-hydroxyphenylpyruvate dioxygenase. WO 96/38567 A2 addresses the utility that would be attached to a DNA sequence of a *p*-hydroxyphenylpyruvate dioxygenase gene, but there is no biochemical evidence of function associated with the sequences disclosed.

SUMMARY OF THE INVENTION

This invention pertains to the isolation and characterization of nucleic acid fragments encoding plant *p*-hydroxyphenylpyruvate dioxygenase enzymes. More specifically, this invention pertains to isolated nucleic acid fragments encoding the *p*-hydroxyphenylpyruvate dioxygenase enzymes from *Arabidopsis thaliana* and *Zea mays*.

This invention also pertains to the production of active plant *p*-hydroxyphenylpyruvate dioxygenase enzyme in *E. coli*. In one embodiment, a chimeric gene comprising a nucleic acid fragment encoding a polypeptide that possesses *p*-hydroxyphenylpyruvate dioxygenase activity, operably linked to regulatory sequences that direct gene expression in *E. coli*, is claimed. In another embodiment, a plasmid vector comprising said chimeric gene is disclosed. In yet another embodiment, a transformed *E. coli* comprising a chimeric gene consisting of a nucleic acid fragment encoding a polypeptide that possesses *p*-hydroxyphenylpyruvate dioxygenase activity is disclosed.

This invention also pertains to a method of identifying substances that inhibit the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme. In one embodiment, the invention pertains to an assay for the detection of inhibitors of *p*-hydroxyphenylpyruvate dioxygenase wherein a polypeptide

derived from a transformed *E. coli* that displays *p*-hydroxyphenylpyruvate dioxygenase activity is incubated in the presence of a test substance. Following incubation, *p*-hydroxyphenylpyruvate dioxygenase enzymatic activity is measured wherein a reduction of enzymatic activity is indicative of the inhibitory capacity of the test substance. Enzymatic activity can be measured by any appropriate means, including but not limited to oxygen utilization, carbon dioxide release, homogentisate production, and loss of *p*-hydroxyphenylpyruvate. Results are quantified by radiometric, colorimetric or chromatographic means.

In another embodiment, this invention pertains to plants that are substantially tolerant to the application of at least one compound that inhibits the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase. Plants may be rendered tolerant by overexpression of the wild-type *p*-hydroxyphenylpyruvate dioxygenase, by expression of a naturally-occuring resistant variant of this enzyme, or by expression of an altered form of *p*-hydroxyphenylpyruvate dioxygenase that is resistant to the action of compounds that are inhibitory to the wild-type enzyme.

A further embodiment of the invention is an isolated nucleic acid fragment comprising a member selected from the group consisting of:

(a) an isolated nucleic acid fragment as set forth in SEQ ID NO:16;

(b) an isolated nucleic acid fragment that is essentially similar to an isolated nucleic acid fragment as set forth in SEQ ID NO:16; and

(c) an isolated nucleic acid fragment that is complementary to (a) or (b).

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE DESCRIPTIONS

The invention can be more fully understood from the following detailed description and the accompanying drawings and the sequence descriptions which form a part of this application.

Figure 1 presents a partial nucleic acid sequence of an expressed sequence tag (EST) bearing GenBank Accession No. T92052 obtained from an *Arabidopsis thaliana* cDNA library. This sequence was contained in clone 91B13T7 of the library.

Figure 2 presents the nucleic acid sequence of the cloned cDNA encoding a full-length form of *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme, as it was initially determined (SEQ ID NO:2). Translation start and stop codons are underlined. Selected restriction sites are indicated.

Figure 3 presents the amino acid sequence comparison between full-length *p*-hydroxyphenylpyruvate dioxygenases from *Arabidopsis thaliana* (SEQ ID NO:15) and *Zea mays* (SEQ ID NO:11) and the *p*-hydroxyphenylpyruvate dioxygenase enzymes derived from human (SEQ ID NO:6, GenBank Acc. No. U29895), pig (SEQ ID NO:7, GenBank Acc. No. D13390), mouse (SEQ ID NO:8, GenBank Acc. No. D29987) and rat (SEQ ID NO:9, GenBank Acc. No. M18405). Asterisks indicate amino acid residues that are conserved across all six species. This figure was created using the Pileup program of GCG (Program Manual for the Wisconsin Package, Version 9.0-OpenVMS, December 1996, Genetics Computer Group, 575 Science Drive, Madison, WI, USA 53711).

Figure 4 is a diagram describing the construction of the intermediate plasmid vector pT7BlueR + PDO1.

Figure 5 is a diagram describing the construction of *E. coli* expression vector pE24CP1.

Applicants have provided a sequence listing in conformity with "Rules for the Standard Representation of Nucleotide and Amino Acid Sequences in Patent Applications" (Annexes I and II to the Decision of the President of the EPO, published in Supplement No. 2 to OJ EPO, 12/1992) and with 37 C.F.R. 1.821-1.825 and Appendices A and B ("Requirements for Application Disclosures Containing Nucleotides and/or Amino Acid Sequences").

SEQ ID NO:1 presents a partial nucleic acid sequence of an expressed sequence tag (EST) bearing GenBank Accession No. T92052 obtained from an *Arabidopsis thaliana* cDNA library. This sequence was contained in clone 91B13T7 of the library.

SEQ ID NO:2 presents the initial determination of the nucleic acid sequence and the deduced amino acid sequence of a cDNA encoding a full-length form of *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme, as contained in plasmid pGBPPD2.

SEQ ID NO:3 presents the initially deduced amino acid sequence encoded by a cDNA for *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme.

SEQ ID NOS:4 and 5 present the nucleotide sequences of a pair of complementary oligonucleotides (CAM 32 and CAM 33, respectively) used to facilitate subcloning and expression of the gene encoding *p*-hydroxyphenylpyruvate dioxygenase without the chloroplast transit sequence.

SEQ ID NO:6 presents the amino acid sequence of *p*-hydroxyphenylpyruvate dioxygenase enzyme derived from human (GenBank Acc. No. U29895).

SEQ ID NO:7 presents the amino acid sequence of *p*-hydroxyphenylpyruvate dioxygenase enzyme derived from pig (GenBank Acc. No. D13390).

SEQ ID NO:8 presents the amino acid sequence of *p*-hydroxyphenylpyruvate dioxygenase enzyme derived from mouse (GenBank Acc. No. D29987).

SEQ ID NO:9 presents the amino acid sequence of *p*-hydroxyphenylpyruvate dioxygenase enzyme derived from rat (GenBank Acc. No. M18405).

SEQ ID NO:10 presents the nucleic acid sequence and deduced amino acid sequence of the cloned cDNA encoding the *Zea mays p*-hydroxyphenylpyruvate dioxygenase enzyme, as contained in plasmid pMPDO.

SEQ ID NO:11 presents the deduced amino acid sequence of the cloned cDNA encoding the *Zea mays p*-hydroxyphenylpyruvate dioxygenase enzyme, as contained in plasmid pMPDO.

SEQ ID NO:12 presents the nucleic acid sequence and the deduced amino acid sequence of the truncated form of *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme as contained in pE24CP1.

SEQ ID NO:13 presents the deduced amino acid sequence of the truncated form of *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme as contained in pE24CP1.

SEQ ID NO:14 presents the revised nucleic acid sequence and the deduced amino acid sequence of the cloned cDNA encoding the full-length *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme, as contained in plasmid pGBPPD2.

SEQ ID NO:15 presents the revised amino acid sequence deduced from the cDNA for the full length *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme.

SEQ ID NO:16 presents the nucleic acid sequence determined from a portion of a cDNA from *Vernonia galamenensis*, as contained in clone vs1.pk0015.b2.

DETAILS OF THE INVENTION

BIOLOGICAL DEPOSITS

The following biological materials have been deposited under the terms of the Budapest Treaty at American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852, and bear the following accession numbers:

Depositor Identification		Int'l. Depository	
Host Strain	Plasmid	Accession Number	Date of Deposit
E. coli BL21(DE3)	pE24CP1	ATCC 98083	June 25, 1996
N/A	pGBPPD2	ATCC 97622	June 25, 1996
N/A	pMPDO	ATCC 209120	June 12, 1997

Definitions

In the context of this disclosure, a number of terms shall be utilized. As used herein, the term "nucleic acid" refers to a large molecule which can be single-stranded or double-stranded, composed of monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. A "nucleic acid fragment" is a portion of a given nucleic acid molecule. As used herein, "DNA" (deoxyribonucleic acid) is the genetic material, whereas "RNA" (ribonucleic acid) is involved in the transfer of the information encoded by the DNA into proteins and polypeptides. A "genome" is the entire body of genetic material contained in each cell of an organism. The term "nucleotide sequence" refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA polymers.

As used herein, "essentially similar" refers to DNA sequences that may involve base changes that do not cause a change in the encoded amino acid or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. It is therefore understood that the invention encompasses more than the specific exemplary sequences. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce "silent changes" (i.e., those that do not substantially affect the functional properties of the resulting protein molecule) are also contemplated. For example, alteration(s) in the gene sequence which reflects the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated; thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be

expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Moreover, the skilled artisan recognizes that "essentially similar" sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65°C), with the sequences exemplified herein.

"Gene" refers to a nucleic acid fragment that encodes a specific protein, including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. "Native" gene refers to the gene as found in nature with its own regulatory sequences. "Chimeric" gene refers to a gene comprising heterogeneous regulatory and coding sequences. "Endogenous" gene refers to the native gene normally found in its natural location in the genome. A "foreign" gene refers to a gene not normally found in the host organism but that is introduced by gene transfer.

"Coding sequence" refers to a DNA sequence that codes for a specific protein and excludes the non-coding sequences.

"Initiation codon" and "termination codon" refer to a unit of three adjacent nucleotides in a coding sequence that specifies initiation and termination, respectively, of protein synthesis (mRNA translation). "Open reading frame" refers to the amino acid sequence encoded between translation initiation and termination codons of a coding sequence.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript. "Messenger RNA" (mRNA) refers to RNA that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA, one strand of which is complementary to and derived from mRNA by reverse transcription. "Sense RNA" refers to RNA transcript that includes the mRNA.

As used herein, "regulatory sequences" are nucleotide sequences that control the transcription or expression of a coding sequence located upstream (5'), within, or downstream (3') to the coding sequence, act in conjunction with the protein biosynthetic apparatus of the cell and include promoters, translation leader sequences, transcription termination sequences, and polyadenylation sequences.

"Promoter" refers to a DNA sequence in a gene, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions. In the case of eukaryotic organisms, it may also contain enhancer elements.

An "enhancer element" is a DNA sequence which can stimulate promoter activity. It may be an innate element of the promoter or a heterologous element inserted to enhance the activity level and tissue-specificity of a promoter. "Constitutive promoters" refer to those enhancer elements that direct gene expression in all tissues and at all times. "Organ-specific" or "development-specific" promoters as referred to herein are those that direct gene expression almost exclusively in specific organs, such as leaves or seeds, or at specific development stages in an organ, such as in early or late embryogenesis, respectively.

The term "operably linked" refers to nucleic acid sequences on a single nucleic acid molecule which are associated so that the function of one is affected by the other. For example, a promoter is operably linked with a structural gene (i.e., a gene encoding *p*-hydroxyphenylpyruvate dioxygenase, as disclosed herein) when it is capable of affecting the expression of that structural gene (i.e., that the structural gene is under the transcriptional control of the promoter).

The term "expression", as used herein, is intended to mean the production of the protein product encoded by a gene. More particularly, "expression" refers to the transcription and stable accumulation of the sense RNA (mRNA) derived from the nucleic acid fragment(s) of the invention that, in conjuction with the protein apparatus of the cell, results in altered levels of protein product. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms. "Altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms. "Facilitating expression" refers to steps and conditions for culturing host cells containing the desirable gene to yield an increased production of the enzyme. For example, addition of a chemical inducer specific to the particular promoter operably linked to the gene facilitates expression of the encoded enzyme. This is measured relative to the production levels of an untreated gene.

The "3' non-coding sequences" refers to the DNA sequence portion of a gene that contains a polyadenylation signal and any other regulatory signal capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

The "translation leader sequence" refers to that DNA sequence portion of a gene between the promoter and coding sequence that is transcribed into RNA and is present in the fully processed mRNA upstream (5') of the translation start codon. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability, or translation efficiency.

"Transformation" herein refers to the transfer of a foreign gene into the genome of a host organism and its genetically stable inheritance. Bacterial transformation can proceed by any of several methods well known in the art, including calcium chloride-mediated transformation and electroporation. Examples of methods of plant transformation include *Agrobacterium*-mediated transformation and particle-accelerated or "gene gun" transformation technology (U.S. Patent No. 4,945,050).

"Host cell" refers to the cell that is transformed with the introduced genetic material.

"Plasmid vector" refers to a double-stranded, closed circular, extra-chromosomal DNA molecule.

"Tolerant" or "tolerance" refers to a condition whereby a cell or an organism is able to withstand the effect of application of a compound or composition at a concentration or application rate that causes a demonstrable effect in or against cells or organisms that are not tolerant. For example, the growth or survival of a plant that is tolerant to application of a herbicidal compound or composition will be less affected than the growth or survival of a plant that is not tolerant to application of the herbicidal compound or composition.

Cloning of Plant Genes Encoding *p*-Hydroxyphenylpyruvate Dioxygenase

The *p*-hydroxyphenylpyruvate dioxygenases from plants are a promising new class of targets for new herbicidal compounds. In order to be able to study this enzyme in detail, and to have available supplies of enzyme for inhibitor screening, cDNA clones encoding plant *p*-hydroxyphenylpyruvate dioxygenases were identified. These nucleic acid fragments are useful for the production of their encoded enzymes, for isolation of clones from additional plant sources that encode other *p*-hydroxyphenylpyruvate dioxygenase enzymes, and for understanding the biochemical and structural properties of these enzymes.

Nucleic acid fragments comprising nucleotide sequences that encode different forms of the enzyme *p*-hydroxyphenylpyruvate dioxygenase from the plant *Arabidopsis thaliana* have now been isolated. Subsequently, these nucleotide sequences were expressed in *E. coli* cells and shown to direct the synthesis of plant *p*-hydroxyphenylpyruvate dioxygenase enzymes.

An automated search of nucleotide sequences contained in a database representing an *Arabidopsis* cDNA library for sequences homologous to other known, non-plant *p*-hydroxyphenylpyruvate dioxygenase genes revealed the plasmid cDNA clone 91B13T7. This cDNA was obtained from the Arabidopsis Seed Stock Center at Ohio State University. Plasmid DNA suitable for nucleotide sequence determination was prepared and the nucleotide sequence of the plasmid insert was determined. The resulting sequence was not interpretable, suggesting possible contamination of the plasmid sample by an extraneous nucleic acid. This assumption was confirmed by digesting the plasmid DNA sample with restriction enzymes and separating the resulting nucleic acid fragments by agarose gel electrophoresis. This analysis revealed the presence of nucleic acid fragments that could not be derived from the plasmid carrying the putative *p*-hydroxyphenyl-pyruvate dioxygenase fragment. Furthermore, a search of the publically available nucleic acid sequence databases revealed that the *Arabidopsis thaliana* sequence reported for cDNA clone 91B13T7 corresponded to a truncated cDNA (Figure 1). Based on publically available mammalian cDNA sequence information for *p*-hydroxyphenylpyruvate dioxygenase, the minimum length expected for a cDNA encoding a complete *p*-hydroxyphenylpyruvate dioxygenase enzyme is 1 kb (Table 1).

Table 1

Predicted cDNA Length for Sequences Encoding *p*-Hydroxyphenylpyruvate Dioxygenase

Organism	Amino Acid Residues	Minimum cDNA (kb)
Human	392	1.176
Pig	392	1.176
Pseudomonas sp.	357	1.071

Therefore, based on the expected length of a cDNA capable of encoding a functional *p*-hydroxyphenylpyruvate dioxygenase, the *Arabidopsis thaliana* sequence obtained from the public database was insufficient to encode a full-length, active *p*-hydroxyphenylpyruvate dioxygenase enzyme. Therefore, a cDNA with the capacity to encode a full-length enzyme *Arabidopsis thaliana* was cloned,

as described herein. A 400 bp segment of the insert of plasmid 91B13T7 was liberated by digestion with restriction enzymes and used to screen a cDNA library prepared from norflurazon-treated *Arabidopsis thaliana* seedlings (Scolnik, P. A., and Bartley, G. E. (1994) *Plant Physiol.* 104:1469-1470). Several clones showing positive hybridization to this probe were sequenced. The initial determination of the sequence of the longest cDNA clone obtained from this effort is shown in Figure 2 and in SEQ ID NO:2. During the course of subsequent work with this clone it became necessary to confirm certain features of the sequence. A corrected sequence of this cDNA is presented in SEQ ID NO:12.

The sequence reported in Figure 2 indicates that this cDNA has the capacity to encode a protein of MW 48,841 which, as shown in Figure 3, has a high level of homology to *p*-hydroxyphenylpyruvate dioxygenase enzymes from other eukaryotes.

A cDNA capable of encoding a full-length *p*-hydroxyphenylpyruvate dioxygenase has also been obtained from corn. This cDNA, contained in plasmid pMPDO, was identified in a corn cDNA library using an approximately 900 base pairs portion of the *Arabidopsis* cDNA as a probe. The predicted amino acid sequence that is encoded by the corn cDNA is also compared to *p*-hydroxyphenylpyruvate dioxygenase enzymes from other eukaryotes in Figure 3.

A cDNA library was prepared from messenger RNA isolated from developing seeds of *Vernonia galamenensis*. Random sequencing of the clones contained in the library identified a probable clone, designated vs1.pk0015.b2, for the *p*-hydroxyphenylpyruvate dioxygenase from this plant. The 513 bp expressed sequence tag (EST) is presented in SEQ ID NO:16.

<u>Expression of the *Arabidopsis thaliana* cDNA Encoding *p*-Hydroxyphenylpyruvate Dioxygenase in *E. coli*</u>

The nucleic acid fragments of the instant invention encoding a plant *p*-hydroxyphenylpyruvate dioxygenase enzymes can be operably linked to suitable regulatory sequences, thereby creating chimeric genes that can be used to direct expression of the enzyme in transgenic organisms. These transgenic organisms include, but are not limited to: plants (*Plant Molecular Biology*; Croy, R. R. D., Ed.; Bios Scientific Publishers; 1993); microorganisms, including *Escherichia coli* (Gold, L. (1990) *Methods in Enzymology* 185:11), *Bacillus subtilis* (Henner, D. J. (1990) *Methods in Enzymology* 185:199), yeast (Gellissen, G., et al. (1992) *Antonie Leeuwenhoek* 62:79), and fungi, including members of the genus *Aspergillus* (Devchand, M. and Gwynne, D. I. (1991) *J. Biotechnol.* 17:3); and insect cells containing recombinant baculoviruses (Lukow, V. A. and Summers, M. D. (1988) *Bio/Technology* 6:47).

One skilled in the art can isolate the coding sequences from the fragments of the invention by using or creating sites for restriction endonucleases, as described in Sambrook, J., et al.((1989) *Molecular Cloning, A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press; hereinafter "Maniatis"). Alternatively, polymerase chain reaction (PCR) techniques can be employed to isolate and/or modify the fragments of the invention (Newton, C. R. and Graham, A. (1994) *PCR*; Bios Scientific Publishers).

Arabidopsis *p*-hydroxyphenylpyruvate dioxygenase was expressed in *E. coli* under control of a T7 promoter in a strain expressing T7 RNA polymerase (Studier, F. W., et al. (1990) *Methods in Enzymology* 185:60). Promoters other than T7 are commonly used in expression vectors and could be substituted for protein expression in *E. coli*. Examples of alternative promoters include, but are not limited to, *trp* (Yansura, D. G. and Henner, D. J. (1990) *Methods in Enzymology* 185:54), P_L (Remaut, E. et al. (1981) *Gene* 15:81), *tac* (Amann, E. et al. (1983) *Gene* 25:167), *trc* (Amann, E. et al. (1988) *Gene* 69:301), and promoters such as *lacUV5*, *lpp*, P_R, and hybrid and tandem promoters constructed to combine specific features to increase strength or regulation capacity (Balbas, P. and Bolivar, F. (1990) *Methods in Enzymology* 185:14).

Biochemical Evidence of Enzymatic Function

The enzyme *p*-hydroxyphenylpyruvate dioxygenase catalyzes the reaction of *p*-hydroxyphenylpyruvate with molecular oxygen to give homogentisate and CO_2. The enzyme can be assayed by measuring oxygen utilization (Hager, S. E., et al. (1957) *J. Biol. Chem.* 225:935-947), CO_2 release or homogentisate production from radioactive labeled *p*-hydroxyphenylpyruvate (Lindblad, B. (1971) *Clin. Chem. Acta* 34:113-121), loss of the *p*-hydroxyphenylpyruvate (Lin, E. C. C. et al. (1958) *J. Biol. Chem.* 233:668-673), or formation of homogentisate using a colorimetric assay (Fellman, J. H. et al. (1972) *Biochim. Biophys. Acta* 284:90-100) or UV detection following HPLC or a similar chromatographic separation technique. The activity of *p*-hydroxyphenylpyruvate dioxygenase may also be measured in a coupled assay in which the initial product, homogentisate, is oxidized by homogentisate dioxygenase: formation of maleylacetoacetate determined by measuring absorbance at 330 nm (Fernández-Cañón, J. M. and Peñalva, M. A. (1997) *Anal. Biochem.* 245:218-221).

An alternative to any of the kinetic assays for *p*-hydroxyphenylpyruvate dioxygenase is an end-point or fixed-time assay. The procedure is based on the conversion of unconverted substrate, *p*-hydroxyphenylpyruvate to its enediol tautomer by tautomerase in the presence of borate ions and measurement of the characteristic 308 nm peak of the tautomer (Lin, E. C. C. et al. (1958) *J. Biol.*

Chem. 233:668-673). The procedure involves the addition of enough *p*-hydroxyphenylpyruvate dioxygenase to consume ~80% of the organic substrate over 1 hour in 200 µL of assay buffer, which in this case is a 50 mM Tris, pH 7.4, 0.10 mM *p*-hydroxyphenylpyruvic acid, 1.75 mM ascorbate and 1.25 mM EDTA. After 1 hr the reaction is quenched by the addition of 100 µL of 0.8 M borate, pH 7.3, containing 1000 ppb of a *p*-hydroxyphenylpyruvate dioxygenase inhibitor and 0.25 µL of 6.1 mg/mL of tautomerase. The absorbance at 308 nm is read after a 30 min incubation and is stable thereafter for 2 hr. The advantage of this assay over the kinetic procedure is that the *p*-hydroxyphenylpyruvate dioxygenase is not required to oxidize the substrate in the presence of high concentrations of borate, a condition that might interfere with the mode of action of inhibitors. Furthermore the assay produces essentially a stable binary indication of *p*-hydroxypheny-lpyruvate dioxygenase inhibition, and is well-suited for applications which require a high-throughput of samples and assays.

The enzyme encoded by the nucleic acid fragments and overexpressed in *E. coli* can be extracted in any conventional buffer used for extracting soluble plant enzymes. Although a large amount of an overexpressed protein is often insoluble, the amount that is soluble represents can represent as much as 50% of the total soluble protein. Soluble overexpressed protein has high *p*-hydroxyphenylpyruvate dioxygenase activity and is easily extracted. Likewise, it may be possible to resolubilize an insoluble overexpressed protein in an active form under appropriate conditions, since addition of sarkosyl (sodium N-lauroylsarcosinate) to the extraction buffer appeared to increase the amount of the overexpressed protein extracted. For optimum activity, a reducing agent such as ascorbate or reduced glutathione should be present as well as a source a ferrous ion.

An overexpressed enzyme can be assayed using all the techniques described above for measuring *p*-hydroxyphenylpyruvate dioxygenase activity, while only the techniques using labeled *p*-hydroxyphenylpyruvate can be used to measure activity in crude plant extracts. Therefore, the availability of an overexpressed enzyme greatly facilitates the development of high capacity screens to identify inhibitors of the enzyme. Potential inhibitors are evaluated for their capacity to reduce the rate of the reaction of the enzyme, resulting in reduced oxygen uptake and CO_2 release, and lower rates of formation of homogentisate and loss of *p*-hydroxyphenylpyruvate. Applicants have demonstrated that at least one of the instant nucleic acid fragments can be overexpressed in *E. coli* cells, resulting in production of a protein that catalyzes the conversion of *p*-hydroxyphenylpyruvate to homogentisate with the release of CO_2. Furthermore, it has been shown that this activity is inhibited by commercial herbicides known to

inhibit *p*-hydroxyphenylpyruvate dioxygenase. Finally, an overexpressed enzyme can be used in a high capacity assay to identify compounds that inhibit the enzymatic activity of *p*-hydroxyphenylpyruvate dioxygenase. Such compounds may serve as herbicides.

Preparation of Plants Tolerant to Inhibitors of *p*-Hydroxyphenylpyruvate Dioxygenase

This invention embodies plants which are resistant or at least tolerant to herbicides that target the *p*-hydroxyphenylpyruvate dioxygenase enzyme at levels which are normally inhibitory to the naturally occurring *p*-hydroxyphenylpyruvate dioxygenase enzyme. This altered *p*-hydroxyphenylpyruvate dioxygenase activity is conferred by (1) overexpression of the wild-type *p*-hydroxyphenylpyruvate dioxygenase enzyme, or (2) expression of a DNA molecule encoding a herbicide-tolerant enzyme. The said enzyme may be a modified form of an *p*-hydroxyphenylpyruvate dioxygenase enzyme that occurs naturally in a eukaryote or prokaryote, or a modified form of an *p*-hydroxyphenylpyruvate dioxygenase enzyme that naturally occurs in a plant, or a herbicide tolerant enzyme that naturally occurs in a prokaryote (Duke et al. *Herbicide Resistant Crops*; Lewis: Boca Raton:1994). An effective amount of gene expression to render the cells of the plant tissue substantially tolerant to the herbicide depends on whether the gene codes for an unaltered *p*-hydroxyphenylpyruvate dioxygenase gene or a mutant or altered form of the gene that is less sensitive to the herbicides. Expression of an unaltered plant *p*-hydroxyphenylpyruvate dioxygenase gene in an effective amount is that amount that provides for a 2- to 10-fold increase in herbicide tolerance. Plants encompassed by the invention include monocotyledoneous and dicotyledoneous plants. Preferred are those plants which would be potential targets for *p*-hydroxyphenylpyruvate dioxygenase-inhibiting herbicides, particularly agronomically important crops such as maize and other cereal crops.

Increased levels of expression of *p*-hydroxyphenylpyruvate dioxygenase activity, from two to ten or more times the natively expressed amount, would be sufficient to overcome growth inhibition caused by the herbicide. Plants containing such altered *p*-hydroxyphenylpyruvate dioxygenase enzyme activity can be obtained by direct selection in plants. This method is known in the art. See, e.g., U.S. Patent No. 5,162,602, U.S. Patent No. 4,761,373, and references cited therein.

Overexpression of *p*-hydroxyphenylpyruvate dioxygenase also can be accomplished by stably transforming a host plant cell with a chimeric DNA molecule comprising a promoter capable of driving expression of an associated coding sequence in a plant cell and operably linked to a homologous or

heterologous coding sequence encoding *p*-hydroxyphenylpyruvate dioxygenase. A "homologous" *p*-hydroxyphenylpyruvate dioxygenase gene is isolated from an organism taxonomically identical to the target plant cell, whereas a "heterologous" *p*-hydroxyphenylpyruvate dioxygenase gene is obtained from an organism taxonomically distinct from the target plant.

The expression of foreign genes in plants is well-established (De Blaere et al., (1987) *Meth. Enzymol.* 143:277-291). Promoters utilized to drive gene expression in transgenic plants or plant cells (i.e., those capable of driving expression of the associated coding sequences such as *p*-hydroxyphenylpyruvate dioxygenase in plant cells, include those directing the 19S and 35S transcripts in Cauliflower mosaic virus (Odell et al., (1985) *Nature* 313:810-812; Hull et al., (1987) *Virology* 86:482-493), small subunit of ribulose 1.5-bisphosphate carboxylase (Morelli et al., (1985) *Nature* 315:200-204; Broglie et al., (1984) *Science* 224:838-843; Herrera-Estrella et al., (1984) *Nature* 310:115-120; Coruzzi et al., (1984) *EMBO J.* 3:1671-1679; Faciotti et al., (1985) *Bio/Technology* 3:241 and chlorophyll a/b binding protein (Lamppa et al., (1986) *Nature* 316:750-752); nopaline synthase promoters (Depicker et al. (1982) *J. Mol. App. Genet. 1*:561-573; An et al. (1990) *Plant Cell 2*:225-233). The chimeric DNA construct(s) of the invention may contain multiple copies of a promoter or multiple copies of the *p*-hydroxyphenylpyruvate dioxygenase coding sequences. In addition, the construct(s) may include coding sequences for selectable markers and coding sequences for other peptides such as signal or transit peptides. The preparation of such constructs is within the ordinary level of skill in the art. Resistance to inhibitors of the plant carotenoid biosynthesis pathway, which is also targeted by *p*-hydroxyphenylpyruvate dioxygenase inhibitors, has been achieved by expressing a bacterial gene encoding phytoene desaturase driven by the CaMV promoter (Misawa et al., (1994) *Plant. J.* 4:481-490).

Transit peptides may be fused to the *p*-hydroxyphenylpyruvate dioxygenase coding sequence in the chimeric DNA constructs of the invention to direct transport of the expressed *p*-hydroxyphenylpyruvate dioxygenase enzyme to the desired site of action. Examples of transit peptides include the chloroplast transit peptides such as those described in Von Heijne et al., (1991) *Plant Mol. Biol. Rep.* 9:104-126; Mazur et al., (1987) *Plant Physiol.* 85:1110; Vorst et al., (1988) *Gene* 65:59; and mitochondrial transit peptides such as those described in Boutry et al., (1987) *Nature* 328:340-342.

It is envisioned that the introduction of enhancers or enhancer-like elements into other promoter constructs will also provide increased levels of primary transcription to accomplish the invention. These would include viral enhancers

such as that found in the 35S promoter (Odell et al., (1988) *Plant Mol. Biol.* 10:263-272), enhancers from the opine genes (Fromm et al., (1989) *Plant Cell* 1:977-984), or enhancers from any other source that result in increased transcription when placed into a promoter operably linked to the nucleic acid fragment of the invention.

Introns isolated from the maize Adh-1 and Bz-1 genes (Callis et al., (1987) *Genes Dev.* 1:1183-1200), and intron 1 and exon 1 of the maize Shrunken-1 (sh-1) gene (Maas et al., (1991) *Plant Mol. Biol.* 16:199-207) may also be of use to increase expression of introduced genes. Results with the first intron of the maize alcohol dehydrogenase (Adh-1) gene indicate that when this DNA element is placed within the transcriptional unit of a heterologous gene, mRNA levels can be increased by 6.7-fold over normal levels. Similar levels of intron enhancement have been observed using intron 3 of a maize actin gene (Luehrsen, K. R. and Walbot, V., (1991) *Mol. Gen. Genet.* 225:81-93). Enhancement of gene expression by Adh1 intron 6 (Oard et al., (1989) *Plant Cell Rep* 8:156-160) has also been noted. Exon 1 and intron 1 of the maize sh-1 gene have been shown to individually increase expression of reporter genes in maize suspension cultures by 10 and 100-fold, respectively. When used in combination, these elements have been shown to produce up to 1000-fold stimulation of reporter gene expression (Maas et al., (1991) *Plant Mol. Biol.* 16:199-207).

Any 3' non-coding region capable of providing a polyadenylation signal and other regulatory sequences that may be required for proper expression can be used to accomplish the invention. This would include the 3' end from any storage protein such as the 3' end of the 10kd, 15kd, 27kd and alpha zein genes, the 3' end of the bean phaseolin gene, the 3' end of the soybean β-conglycinin gene, the 3' end from viral genes such as the 3' end of the 35S or the 19S cauliflower mosaic virus transcripts, the 3' end from the opine synthesis genes, the 3' ends of ribulose 1,5-bisphosphate carboxylase or chlorophyll a/b binding protein, or 3' end sequences from any source such that the sequence employed provides the necessary regulatory information within its nucleic acid sequence to result in the proper expression of the promoter/coding region combination to which it is operably linked. There are numerous examples in the art that teach the usefulness of different 3' non-coding regions (for example, see Ingelbrecht et al., (1989) *Plant Cell* 1:671-680).

Various methods of introducing a DNA sequence (i.e., of transforming) into eukaryotic cells of higher plants are available to those skilled in the art (see EPO publications 0 295 959 A2 and 0 138 341 A1). Such methods include high-velocity ballistic bombardment with metal particles coated with the nucleic acid

constructs (see Klein et al., (1987) *Nature* (London) 327:70-73. and see U.S. Patent No. 4,945,050), as well as those based on transformation vectors based on the Ti and Ri plasmids of *Agrobacterium* spp., particularly the binary type of these vectors. Ti-derived vectors transform a wide variety of higher plants, including monocotyledonous and dicotyledonous plants, such as soybean, cotton and rape seed (Pacciotti et al., (1985) *Bio/Technology* 3:241; Byrne et al., (1987) *Plant Cell, Tissue and Organ Culture* 8:3; Sukhapinda et al., (1987) *Plant Mol. Biol.* 8:209-216; Lorz et al., (1985) *Mol. Gen. Genet.* 199:178-182; Potrykus et al., (1985) *Mol. Gen. Genet.* 199:183-188).

Other transformation methods are available to those skilled in the art, such as direct uptake of foreign DNA constructs (see EPO publication 0 295 959 A2), and techniques of electroporation (see Fromm et al., (1986) *Nature* (London) 319:791-793). Once transformed, the cells can be regenerated by those skilled in the art. Also relevant are several recently described methods of introducing nucleic acid fragments into commercially important crops, such as rapeseed (see De Block et al., (1989) *Plant Physiol.* 91:694-701), sunflower (Everett et al., (1987) *Bio/Technology* 5:1201-1204), soybean (McCabe et al., (1988) *Bio/Technology* 6:923-926; Hinchee et al., (1988) *Bio/Technology* 6:915-922; Chee et al., (1989) *Plant Physiol.* 91:1212-1218; Christou et al., (1989) *Proc. Natl. Acad. Sci USA* 86:7500-7504; EPO Publication 0 301 749 A2), and corn (Gordon-Kamm et al., (1990) *Plant Cell* 2:603-618; and Fromm et al., (1990) *Bio/Technology* 8:833-839).

Altered *p*-hydroxyphenylpyruvate dioxygenase enzyme activity may also be achieved through the generation or identification of modified forms of the isolated eukaryotic *p*-hydroxyphenylpyruvate dioxygenase coding sequence having at least one amino acid substitution, addition or deletion which encodes an altered *p*-hydroxyphenylpyruvate dioxygenase enzyme resistant to a herbicide that inhibits the unaltered, naturally occurring form. Genes encoding such enzymes can be obtained by numerous strategies known in the art. A first general strategy involves direct or indirect mutagenesis procedures on microbes (e.g., *E. coli*, *S. cerevisiae* (Miller, (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Davis et al., (1980) Advanced Bacterial Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Sherman et al., (1983) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Gold Spring Harbor NY; and U.S. Patent No. 4,975,374) and cyanobacteria (Bryant, *The Molecular Biology of Cyanobacteria*; Kluwer Academic Publishers: Boston, 1995). A second method of obtaining mutant herbicide-resistant alleles of the eukaryotic *p*-hydroxyphenylpyruvate dioxygenase

enzyme involves direct selection in plants. For example, the effect of inhibitors on the growth of plants such as Arabidopsis, soybean, or maize may be determined by plating seeds sterilized by art-recognized methods on plates on a simple minimal salts medium containing increasing concentrations of the inhibitor. The lowest dose at which significant growth inhibition can be reproducibly detected is used for subsequent experiments. Mutagenesis of plant material may be utilized to increase the frequency at which resistant alleles occur in the selected population. Mutagenized seed material can be derived from a variety of sources, including chemical or physical mutagenesis or seeds, or chemical or physical mutagenesis or pollen (Neuffer, In Maize for Biological Research. Sheridan, ed. Univ. Press, Grand Forks, ND., pp. 61-64 (1982)), which is then used to fertilize plants and the resulting M1 mutant seeds collected. Typically, for Arabidopsis, M2 seeds (i.e., progeny seeds of plants grown from seeds mutagenized with chemicals, such as ethyl methane sulfonate, or with physical agents, such as gamma rays or fast neutrons) are plated at densities of up to 10,000 seeds/plate (10 cm diameter) on minimal salts medium containing an appropriate concentration of inhibitor. Seedlings that continue to grow and remain green 7-21 days after plating are transplanted to soil and grown to maturity and seed set. Progeny of these seeds are tested for resistance to the herbicide. If the resistance trait is dominant, plants whose seed segregate 3:1 (resistant:sensitive) are presumed to have been heterozygous for the resistance at the M2 generation. Plants that give rise to all resistant seed are presumed to have been homozygous for the resistance at the M2 generation. Such mutagenesis on intact seeds and screening of their M2 progeny seed can also be carried out on other species, for instance soybean (see, e.g., U.S. Patent No. 5,084,082). Mutant seeds to be screened for herbicide tolerance can also be obtained as a result of fertilization with pollen mutagenized by chemical or physical means.

EXAMPLE 1

Cloning of a cDNA for *Arabidopsis thaliana* *p*-Hydroxyphenylpyruvate Dioxygenase

The plasmid containing the *Arabidopsis thaliana* 91B13T7 expressed sequence tag (Newman et al., (1994) *Plant Physiol* 106:1241-1255) was digested with the restriction enzymes *Bam*HI and *Eco*RI, and the resulting 400 bp fragment was used to screen a lambda phage cDNA library of *Arabidopsis thaliana* seedlings (Scolnik, P. A. and Bartley, G. E. (1994) *Plant Physiol.* 104:1469-1470) according to the following protocol.

E. coli KW251 cells were grown overnight in Luria Broth ("LB") containing 0.2% maltose and 10 mM $MgSO_4$. Cells were pelleted by centrifugation and

resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5. Cell aliquots (0.8 mL) were mixed with 0.1 mL of diluted phage samples and 7 mL of top agarose (0.7% agarose in LB containing 10 mM $MgSO_4$) at 45°C, and plated onto 150 mm Petri dishes containing LB agar. Phage plaques became visible in 5-7 h, at which point the plates were placed at 4°C.

Phage plaques were transferred to nitrocellulose filters according to standard techniques, and the filters were hybrized to ^{32}P-radiolabeled probe prepared according to the method of Feinberg and Vogelstein ((1983) *Anal. Biochem.* 132:6-13), using the hybridization conditions of Berlyn et al.((1989) *Proc. Natl. Acad. Sci.* 86:4604-4608). After exposure to X-ray film for 48 h, 12 positive plaques were eluted, plated, and hybridized under the same conditions. A total of 9 plaques that retained positive signals in this second round of hybridization were subjected to *in vivo* excision using the Exassist/SOLR™ system according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). DNA from the plasmids resulting from *in vivo* excision of positive plaques was prepared for DNA sequencing using the Wizard Plus™ kit (Promega, Madison, WI). Eight of the clones that were sequenced showed strong conservation with available *p*-hydroxyphenylpyruvate dioxygenase sequences, whereas the remaining clone did not correspond to a *p*-hydroxyphenylpyruvate dioxygenase. Alignment with known *p*-hydroxyphenylpyruvate dioxygenase sequences also revealed that two of the clones correspond to 0.3 kbp fragments from the 3' end of the transcript, and another two to 1.2 kbp fragments from the 5' end of the transcript. One clone of each was used to assemble a 1.5 kbp cDNA by ligating at the internal *Nhe*I restriction site (Figure 1). The initial determination of the DNA sequence (SEQ ID NO:2) of the resulting cDNA clone is shown in Figure 2. Subsequent work with this DNA fragment required confirmation of some of the features of its sequence. Approximately ten nucleotide residues were found to have been listed in error. Thus a corrected sequence for this DNA fragment is listed in SEQ ID NO:14 and the deduced amino acid sequence is set forth in SEQ ID NO:15. The revised sequences form the bases for analyses and comparisons reported herein.

EXAMPLE 2

Overexpression of the *Arabidopsis* cDNA in *E. coli*

The deduced amino acid sequence for *Arabidopsis p*-hydroxyphenylpyruvate dioxygenase was aligned with the amino acid sequences of *p*-hydroxyphenylpyruvate dioxygenase from mouse, pig, and *Streptomyces avermitilis* using the Pileup program of GCG (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, WI, USA 53711). This analysis suggested an additional

29 amino acid-extension at the amino terminus of the *Arabidopsis* sequence (positions 1-29, Figure 3 and SEQ ID NO:3). This amino-terminal extension was assumed to be a chloroplast transit peptide which would be absent from the mature enzyme. Therefore, removal of the chloroplast transit peptide coding sequence coincided with transfer of the *p*-hydroxyphenylpyruvate dioxygenase coding sequence from the cloning vector into the expression vector.

The *Arabidopsis* *p*-hydroxyphenylpyruvate dioxygenase cDNA was moved from the pBluescript SK- cloning vector (Stratagene, La Jolla, CA) to the pET24c(+) expression vector (Novagen, Madison, WI) through the intermediate cloning vector pT7BlueR (Novagen). The plasmid pGBPPD2 consists of the *Arabidopsis* *p*-hydroxyphenylpyruvate dioxygenase cDNA and the pBluescript SK- cloning vector (Stratagene). The plasmid pE24CP1 consists of the *Arabidopsis* *p*-hydroxyphenylpyruvate dioxygenase cDNA, without the putative chloroplast transit peptide DNA sequence, and the pET24c(+) expression vector (Novagen).

The plasmids pGBPPD2 and pT7BlueR (5 μg each) were individually digested with 20 units of Xba I (New England Biolabs, NEB, Beverly, MA) and 20 units of Hind III (Gibco BRL, Gaithersburg, MD) in NEB restriction enzyme buffer 2 supplemented with 100 μg/mL bovine serum albumin at 37 °C for 1.75 h. Digesting pGBPPD2 with the restriction enzymes Xba I and Hind III releases the 5' and 3' ends, respectively, of the *p*-hydroxyphenylpyruvate dioxygenase cDNA from the pBluescript SK- polylinker. Products of the digestion were electrophoretically separated in a 1 percent agarose gel using TRIS/acetate/EDTA (TAE) buffer and visualized with ethidium bromide staining (Maniatis). Digestion of pGBPPD2 with the two restriction endonucleases resulted in a 2922 bp vector band and 1499 bp *p*-hydroxyphenylpyruvate dioxygenase cDNA band. Only a 2863 bp band was apparent after digesting pT7BlueR with the two enzymes, although a 24 bp fragment would also result. The 1499 bp *p*-hydroxyphenylpyruvate dioxygenase band and the 2863 bp T7BlueR band were cut out of the gel and the associated DNA purified from the agarose using a QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. The purified DNA samples were precipitated by the addition of sodium acetate (pH 5.2) to 0.3 M, 10 μg tRNA (added as carrier), two volumes of -20 °C ethanol and incubation at -20 °C overnight. Nucleic acid pellets were collected by centrifugation, washed with 70% ethanol and air dried. Both pellets were solublized in 10 μL of TRIS/EDTA (TE) buffer, pH 8 (Maniatis), and then 1 μL of each sample loaded onto a 1% agarose, TAE gel in separate wells next to a well containing 4 μL of Mass Ladder (Gibco BRL). All samples were adjusted

to 10 μL with water before loading. DNA was quantified by comparing band intensities of each sample with Mass Ladder band intensities following ethidium bromide staining and UV illumination.

Approximately 300 ng of *p*-hydroxyphenylpyruvate dioxygenase insert was mixed with 300 ng of double digested pT7BlueR vector in a total volume of 7 μL and then heated to 45 °C for 5 min followed by cooling on ice. T4 DNA ligase buffer (Gibco BRL) and 1 unit of T4 DNA ligase (Gibco BRL) were added to the cooled DNA for a total volume of 10 μL. The ligation mix was incubated at room temperature for 4 h and then transformed into MAX Efficiency DH5α Competent Cells (Gibco BRL) of *E. coli* according to standard procedures (Maniatis). Transformed bacteria were spread onto LB agar plates supplemented with 100 μg/mL carbenicillin and incubated overnight at 37 °C. Seventeen bacterial colonies were selected for subsequent analysis. A portion of each colony was inoculated into a separate 17x100 mm polypropylene culture tube (Falcon, Lincoln Park, NJ) containing 2 mL of liquid LB media and 200 μg/mL carbenicillin. Liquid bacteria cultures were incubated overnight at 37 °C with shaking (250 rpm). Plasmid DNA was then isolated using a QIAprep Spin Plasmid Miniprep Kit (Qiagen) according to the manufacturer's instructions. A portion (5 μL out of 50 μL total) of each plasmid preparation was digested with 10 units each of Hind III and EcoR V (Gibco BRL) in a total volume of 15 μL with React 2 buffer (Gibco BRL) for one h. (Note: The EcoRV site in the pBluescript polylinker was destroyed during the preparation of pGBPPD2 so only the EcoRV site in the pT7BlueR polylinker would be accessible to the restriction nuclease). Samples were separated electrophoretically in 1% agarose and tris/borate/EDTA (TBE) buffer (Maniatis). Bands were visualized with ethidium bromide staining; 7 out of 17 samples which contained 2 bands (2837 and 1525 bp) contained the *p*-hydroxyphenylpyruvate dioxygenase insert and were designated pT7BlueR+PDO1 (see Figure 4).

In order to remove the putative chloroplast transit sequence, the remaining 45 μL of each prep of pT7BlueR+PDO1 were combined into a single sample and the DNA content determined spectrophotometrically at A_{260} (Maniatis). A portion (5 μg) of pT7BlueR+PDO1 was digested with 16 units of Eco47 III (MBI Fermentas) in a total volume of 100 μL containing buffer 0 (MBI Fermentas) at 37 °C for 2 h. The digested plasmid DNA was then precipitated with sodium acetate and ethanol as above and the resulting dried nucleic acid pellet was dissolved in 60 μL of React 2 (Gibco BRL) containing 20 units of Nde I (Gibco BRL) and incubated 2 h at 37 °C. The double digested sample was then loaded onto a 1% agarose gel in TAE and the large 4166 bp Nde I-Eco47III fragment

separated from the 196 bp fragment electrophoretically. The large fragment was cut out of the gel, purified from agarose and precipitated as above.

An oligonucleotide mix was prepared consisting of 100 pmoles each of oligos CAM32 and CAM33 (SEQ ID NOS:4 and 5, respectively) in a combined volume of 9.9 μL. The two oligos complement each other to form a 3' blunt end corresponding to the 5' half of an Eco47 III restriction site and also form a 5' staggered end which corresponds to the 3' half of an Nde I restriction site.

CAM 32: (SEQ ID NO:4)
5'-TATGTCCAAGTTCGTAAGAAAGAATCCAAAGTCTGATAAATTCAAGGTTAAGC-3'

CAM 33: (SEQ ID NO:5)
5'-GCTTAACCTTGAATTTATCAGACTTTGGATTCTTTCTTACGAACTTGGACA-3'

The oligo mix was heated to 90 °C for 1.5 min and then allowed to cool to room temperature over 20 min. The dried nucleic acid pellet resulting from purification of the 4166 bp Nde I-Eco47 III fragment was solublized in 7 μL of the cooled oligo mix and subsequently heated to 45 °C for 5 min followed by cooling on ice. Ligation of the oligos with the Nde I-Eco47 III fragment followed by transformation into DH5α was performed as above. Transformed bacterial cells were spread onto LB/carbenicillin plates and incubated at 37 °C overnight. Seventeen colonies were selected and processed to isolate plasmid DNA as above. A portion (5 out of 50 μL) of each plasmid was double digested with 10 units each of Nde I and Hind III and the fragments separated electrophoretically on a 1% agarose gel in TBE. A two band pattern corresponding to insert (1373 or 1518 bp) and vector (2844 bp) was detected. An additional double digest with 10 units each of Xba I and Hind III was performed on another 5 μL aliquot of plasmids. When digested with Nde I and Hind III, none of the plasmids which contained the smaller insert size contained a Xba I site. The Xba I site would be eliminated if the two oligos replaced the 196 bp fragment originally present in pT7Blue+PDO1. The 7 plasmid samples with the modified *p*-hydroxyphenylpyruvate dioxygenase insert were combined and designated pT7BlueR+PDO2.

The pT7BlueR+PDO2 plasmid DNA was quantified spectrophotometrically (above) and then 5 μg was digested with 20 units each of Hind III and Nde I in 62 μL of React 2 for 2 h at 37 °C. The digested sample was subsequently loaded onto a 1% agarose gel in TAE and separated electrophoretically. The 1373 bp fragment was isolated and precipitated as above. The plasmid pET24c(+) (5 μg) was double digested with 20 units each of both Nde I and Hind III in React 2 at 37 °C for 2 h and the 5245 bp fragment then gel purified on a 1% agarose gel in

TAE and subsequently separated from agarose and precipitated as above. The dried pET24c(+) pellet was solublized in 10 μL TE and then 8 μL was adjusted to a 20 μL total volume with water, dephosphorylation buffer (Gibco BRL) and 1 unit of calf intestinal alkaline phosphatase (Gibco BRL). The sample was incubated at 37 °C for 30 min and then gel purified, separated from agarose, and precipitated as above. The dried, dephosphorylated, pET24c(+) vector pellet and modified *p*-hydroxyphenylpyruvate dioxygenase insert pellet were each solublized in 10 μL TE and then 1 μL of each was run on a 1% agarose TBE gel with 4 μL of mass ladder to quantify DNA as above. One hundred nanograms of modified *p*-hydroxyphenylpyruvate dioxygenase insert was mixed with 120 ng of dephosphorylated pET24c(+) vector in a total of 7 μL volume. The mix was heated to 45 °C for 5 min and then cooled on ice. The mix was then supplemented with T4 DNA ligase buffer and 1 unit of T4 DNA ligase in a total volume of 10 μL and the mix allowed to incubate at room temperature for 4 h. The ligation mix was subsequently transformed into DH5α, spread on LB agar supplemented with 30 μg/mL kanamycin, and incubated overnight at 37 °C. Plasmid preparations were performed on 11 colonies as above. Plasmids were double digested with Nde I and Hind III and fragments separated electrophoretically. All plasmids had the expected 1373 bp and 5245 bp fragments. One bacteria colony was selected and used to inoculate 100 mL of liquid LB supplemented with 30 μg/mL kanamycin which was subsequently incubated at 37 °C overnight with shaking. Plasmid DNA was isolated from the resulting bacteria culture using a Qiagen Plasmid Midi Kit according to the manufacturer's instructions. A portion of the plasmid DNA (pE24CP1) was sequenced with the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH) using a biotinylated sequencing primer to the T7 promoter (United State Biochemical) according to the manufacturer's instructions for non-radioactive manual sequencing. DNA was transferred from the sequencing gel to Hybond-N+ nylon transfer membrane (Amersham, Arlington Heights, IL) by capillary action. Transfer and all subsequent steps in chemiluminescent detection of DNA fragments were performed with a SEQ-Light Chemiluminescent Sequencing System kit (Tropix, Bedford, MA) according to the manufacturer's instructions. DNA sequencing verified that the plasmid contained the expected 5' sequence for the modified *p*-hydroxyphenylpyruvate dioxygenase insert where nucleotides 1-95 (Figure 2) were replaced with an ATG transcriptional start site. This is equivalent to amino acids 2-29 (Figure 3) being eliminated from the N-terminus of the *Arabidopsis p*-hydroxyphenylpyruvate dioxygenase amino acid sequence.

The plasmid pE24CP1 was transformed into competent cells of BL21(DE3) *E. coli* (Novagen), as above. Transformed cells were spread on LB/kanamycin plates and incubated overnight at 37 °C. Seven colonies were selected for plasmid preparations as above and plasmid DNA was double digested with Nde I and Hind III to verify that all plasmids had the expected electrophoretic banding pattern. One colony was selected and streaked for isolation on LB/kanamycin plates. A well isolated colony was used to inoculate liquid LB supplemented with 30 μg/mL kanamycin and the culture was incubated at 37 °C with shaking (250 rpm) until it reached an A_{600} of 0.6 absorbance units. An 8% glycerol freezer stock was prepared according to the Novagen protocol and stored at -80 °C. All subsequent expression studies were done with freshly grown bacterial cells that were isolated from LB/kanamycin plates streaked from the glycerol freezer stock.

BL21(DE3) *E. coli* cells containing either pE24CP1 or pET24c(+) (negative control) were streaked out onto LB/kanamycin plates from a glycerol freezer stock (above) and incubated overnight at 37 °C. One isolated colony was selected for inoculation of 2 mL of LB containing 30 μg/mL kanamycin in a 17 x 100 mm Falcon tube, and the culture was incubated at 37 °C with shaking (250 rpm) overnight. The overnight cultures were then used to inoculate 100 mL of fresh LB containing 30 μg/mL kanamycin. The new cultures were incubated at 37 °C with shaking until the A_{600} reached between 0.4 and 0.6 absorbance units. One half of the pE24CP1 and pET24c(+) cultures were placed in new culture flasks and IPTG (isopropylthio-β-D-galactoside; Gibco BRL) was added to the new flasks to give a final concentration of 1 mM. The flasks were incubated an additional 3 h at 37 °C with shaking, and then the cells were harvested.

The harvested cells were centrifuged and the resulting cell pellet extracted by sonication (3 x 10 sec bursts) in 2 mL extraction buffer (50 mM (20 mM in the first experiment; Table 2) potassium phosphate buffer, pH 7.2, containing 0.14 M KCl, 0.32 mM reduced glutathione, 1% polyvinylpolypyrrolidone, and 0.1% Triton X 100 (0.01% lysozyme was included in the first experiment only)). The lysate represents the crude extracted enzyme after centrifugation at 17000 g for 10 min. In the first experiment (Table 2) a 20 to 60% ammonium sulfate precipitated enzyme fraction was also assayed. Solid ammonium sulfate was slowly added with stirring to 2 mL of the lysate to bring the concentration to 20% (w/v). After incubation on ice for approximately 15 min, the solution was centrifuged at 17000 g for 10 min. The supernatant liquid was harvested and solid ammonium sulfate was added to increase the concentration to 60% (w/v). After

centrifugation, the resulting pellet was resuspended in 1 mL of the extraction buffer.

A portion of the insoluble protein resulting from expression of *Arabidopsis* *p*-hydroxyphenylpyruvate dioxygenase in bacteria was utilized for N-terminal sequence analysis. The protein (approximately 180 μg) was suspended in 60 μL of extraction buffer and then diluted with 5 volumes of sample buffer (62.5 mM Tris, pH 6.8, 6 M urea, 160 mM dithiothreitol, 0.01% bromophenol blue) followed by intermittent vortexing for one hour at room temperature. A 1.5 mm thick, 12% polyacrylamide resolving gel was prepared for a Mini-Protein II dual slab cell (Bio-Rad, Hercules, CA) using the manufacturer's instructions. The polyacrylamide was allowed to polymerize for 3 h and then a stacking gel was prepared using a preparative comb. The running buffer was prepared according to the manufacturer's instructions with the addition of 0.1 mM sodium thioglycolate. The solublized protein sample was electrophoretically separated using the manufacturer's instructions. When the bromophenol blue dye front reached the bottom of the gel, the gel was removed and equilibrated for 5 min in blotting buffer (10 mM CAPS, pH 11, 10% methanol, balance water). The gel was then placed in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad), according to the manufacturer's instructions, with a ProBlott PVDF membrane (Applied Biosystems, Foster City, CA) treated according to the manufacturer's instruction. Electroblotting was done in the presence of blotting buffer at 50 volts for 45 min in an ice bath. The membrane was then rinsed in water and stained with Coomassie Blue as described in the ProBlott protocol. The major protein band was excised from the membrane and subjected to N-terminal amino acid sequencing on a Beckman (Fullerton, CA) LF3000 protein sequencer. The first 11 cycles identified S-K-F-V-R-K-N-P-K-S-D (see SEQ ID NO:3, amino acids 30-40), respectively. This is the expected N-terminus of the modified *Arabidopsis* *p*-hydroxyphenylpyruvate dioxygenase minus the initial methionine (amino acids 30-40, Figure 3).

EXAMPLE 3

p-Hydroxyphenylpyruvate Dioxygenase Enzymatic Activity of the Plant Protein Expressed in *E. Coli*

Cell cultures with different plasmid constructs were extracted as described above and assayed by measuring the formation of $^{14}CO_2$ from [1-^{14}C]-*p*-hydroxyphenylpyruvate or $^{14}CO_2$ and ^{14}C-homogentisate from [U-^{14}C]-*p*-hydroxyphenylpyruvate (Lindblad, B., (1971) *Clin. Chim. Acta* 34:113-121; and Lindstedt, S. and Odelhog, B., (1987) *Methods in Enzymology* 142:143-148). The labeled substrate was prepared from [1-^{14}C]-L-tyrosine

(55 mCi/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) or [U-^{14}C]-L-tyrosine (498 mCi/mmol; DuPont NEN, Boston, MA). A 50-100 μL aliquot (5-10 μCi) of the of the labeled tyrosine stock solution was transferred to a 4 mL glass vial and blown to dryness in a stream of nitrogen at 45°C. To the vial was added 175 μL of 0.1 M phosphate buffer, pH 6.5, 5 μL catalase (28,700 units of C-100, Sigma Chemical Co., St. Louis, MO), and 20 μL L-amino acid oxidase (Sigma A-9253, 6.5 units/mL). The vial was then placed on a shaker water bath set at 30°C, 60 cycles/min, for 0.5 to 1 h. The reaction mix was then passed through a small column containing 400 μL Dowex AG 50W X8 cation exchange resin. The column was then washed with 1.5 mL of water and the eluant containing the labeled *p*-hydroxyphenylpyruvate was collected. The labeled substrate was either used immediately or stored at -80°C and used within a week after preparation.

The assay was performed in 14 mL culture tubes capped with serum stoppers through which a polypropylene well containing 200 μL of 1 N KOH was suspended. The reaction mixture contained 5,740 units of catalase, 100 μL of a freshly prepared 1:1 (v:v) mixture of 150 mM reduced glutathione and 3 mM dichlorophenolindophenol, 5 mM ascorbate, 0.1 mM ferrous sulfate (the ascorbate and ferrous sulfate were not present in the buffer used in the first experiment; Table 2), 50 μM unlabeled *p*-hydroxyphenylpyruvate, 1-25 μL of the enzyme extract, and 50 mM potassium phosphate buffer in a final volume of 980 μL. Unlabeled substrate was made fresh daily in 50 mM potassium phosphate buffer and allowed to equilibrate for at least 2 h at room temperature to insure that greater than 95% was in the keto form. The tubes were incubated for 10 min at 30°C in a shaking water bath prior to adding 20 μL (0.04 μCi) of ^{14}C-*p*-hydroxyphenylpyruvate. The reaction was terminated after 60 min by injecting 500 μl of 1 N sulfuric acid through the serum stopper. The vials were left on the shaker for another 30 min to insure complete capture of the released $^{14}CO_2$. The serum caps were then removed and the wells cut and dropped into 8 mL scintillation vials. Six mL of Formula-989 scintillation fluid (Packard Insturments, Meriden, CT) was added to the vials and the ^{14}C radioactivity was determined by scintillation counting. Table 2 summarizes the results of this experiment.

Table 2

p-Hydroxyphenylpyruvate Dioxygenase Activity of Extracts from *E. coli* Containing Different Plasmid Constructs

Plasmid	Inducer (1 mM IPTG)	Lysate		Ammonium Sulfate Precipitate	
		dpm * /mg	nmol/min x mg	dpm * /mg	nmol/min x mg
pET24c(+)	-	12,318	0.09	0	0.00
pET24c(+)	+	35,115	0.25	3,393	0.03
pE24CP1	-	24,607	0.17	126,761	0.89
pE24CP1	+	243,801	1.71	1,371,823	9.64

* $^{14}C : ^{12}C = 1 : 50$; sp. act. of ^{14}C-*p*-hydroxyphenylpyruvate = 55 mCi/mmol

The results show there was little or no *p*-hydroxyphenylpyruvate dioxygenase activity in any of the cell cultures that did not have the plasmid containing the nucleic acid fragment encoding *p*-hydroxyphenylpyruvate dioxygenase (pET24c(+)) and the inducer of gene expression (IPTG). The gene and inducer together resulted in a marked increase in activity.

In the experiment with [U-^{14}C] *p*-hydroxyphenylpyruvate ("HPPA"), where both $^{14}CO_2$ and ^{14}C-homogentisic acid were measured, the reaction was initiated by adding 50 μL of labeled substrate (0.3 μCi) and was terminated with 100 μL of 10% phosphoric acid. The $^{14}CO_2$ released was determined by scintillation counting, while the level of homogentisic acid was determined by HPLC on a Zorbax RX-C8 column (4.6 x 250 mm) with an in-line radioactivity detector. Aliquots of 1.7 to 15 μL were taken from the reaction mix after centrifugation and diluted into the column equilibration buffer prior to injection. Separation was performed at ambient temperature with a flow rate of 1.0 mL/min and the following gradient with solvent A and B being water and methanol, each with 1% phosphoric acid: 0-2 min, isocratic at 95% A and 5% B; 2-17 min, linear gradient from 95 to 75% A and 5 to 25% B; 17-19 min linear gradient from 75 to 5% A and 25 to 95% B; 19-22 min, isocratic at 5% A and 95% B; 22-24 min, linear gradient from 5% to 95% A and 95 to 5% B. In this system homogentisate eluted at 10.8 min. The results from this experiment are shown in Table 3.

Table 3

p-Hydroxyphenylpyruvate Dioxygenase Activity of Cell Extracts Determined by CO_2Release and Homogentisic Acid Synthesis from [U-^{14}C] *p*-Hydroxyphenylpyruvate

Plasmid	Inducer (1 mM IPTG)	nmol/min x mg* $^{14}CO_2$	nmol/min x mg* Homogentisic acid
pET24c(+)	-	0.00	0.00
pET24c(+)	+	0.19	0.00
pE24CP1	-	4.68	4.76
pE24CP1	+	29.12	29.82

* ^{14}C : ^{12}C = 1 : 87.7; sp. act. of ^{14}C[U]-*p*-HPPA = 498 mCi /mmol

There was a tight correlation between the results from the assays of the two products of the reaction. The results confirmed there was no significant *p*-hydroxyphenylpyruvate dioxygenase activity in either cell culture that did not contain the nucleic acid fragment encoding *p*-hydroxyphenylpyruvate dioxygenase. There was measureable enzyme activity in the absence of the inducer, but when the inducer was added the activity increased greater than six-fold over uninduced cultures. These results and those of Table 2 clearly show that the nucleic acid fragment isolated and overexpressed in *E. coli* cells encodes a protein that catalyzes the conversion of *p*-hydroxyphenylpyruvate to homogentisate with the release of CO_2.

The overexpressed protein was also assayed spectrophotometrically at ambient temperature using the enol borate-tautomerase assay (Lin, E. C. C. et al., (1958) *J. Biol. Chem.* 233:668-673). The assay buffer contained 0.4 M borate (adjusted to pH 7.2 with 0.2 M sodium borate), 4 mM ascorbate, 2.5 mM EDTA, 40 µM *p*-hydroxyphenylpyruvate, and 0.5 units of tautomerase (Sigma T-6004) per 10 mL buffer. The reaction mix was used when the tautomerization of the substrate was complete (when absorbance at 308 nm had stabilized). The assay was initiated by adding 40 µL of the cell extracts to 960 µL of the assay buffer, and the reaction was followed by measuring the decrease in absorbance at 308 nm. Table 4 summarizes the results with extracts of the same four cell cultures described in Table 3.

Table 4

Spectrophotometric Assay of *p*-Hydroxyphenylpyruvate Dioxygenase Activity of Cell Extracts

Plasmid	Inducer (1 mM IPTG)	nmol *p*-HP lost/min x mg*
pET24c(+)	-	1.58
pET24c(+)	+	2.73
pE24CP1	-	4.91
pE24CP1	+	22.32

* Loss of *p*-hydroxyphenylpyruvate based on a molar extinction coefficient for the equilibrium mixture of 9850 as reported by Lin et al. ((1958) *J. Biol. Chem.* *233*: 668-673).

EXAMPLE 4

Inhibition of *p*-Hydroxyphenylpyruvate Dioxygenase by Commercial Herbicides

The enzymatic activity of the overexpressed protein is inhibited by two herbicides known to inhibit plant *p*-hydroxyphenylpyruvate dioxygenase: Sulcotrione (2-(2-chloro-4-methanesulfonylbenzoyl)-1,3-cyclohexanedione); and Isoxaflutole (5-cyclopropylisoxazol-4-yl 2-mesyl-4-trifluoromethylphenyl ketone). These two compounds were tested against the overexpressed protein using both the $^{14}CO_2$ and the continuous spectrophotometric enol borate-tautomerase assays. Both compounds were added to the assay buffers in 10 µL of acetone or dimethyl sulfoxide. The I_{50} values (concentration inhibiting the enzyme 50%) were calculated based on the percent inhibition observed over several concentrations of the inhibitor. The results of the assays are shown in Table 5.

Table 5

I_{50} Values of Inhibitors of Plant *p*-Hydroxyphenylpyruvate Dioxygenase

Compound	I_{50} value (nM) derived from $^{14}CO_2$ assay	I_{50} value (nM) derived from spectrophotometric assay
sulcotrione	43	44
isoxaflutole	409	1042

These results clearly show that the *p*-hydroxyphenylpyruvate dioxygenase activity of the overexpressed protein is inhibited by commercial herbicides that have inhibition of this enzyme as their mode of action. Moreover, the continuous spectrophotometric assay gave similar I_{50} values to those obtained with the $^{14}CO_2$ assay. The spectrophotometric assay can be adapted to a high capacity screen for

inhibitors of *p*-hydroxyphenylpyruvate dioxygenase by adapting it to a microtiter plate assay combined with a plate reader that would read at or near 308 nm. Furthermore, any colorimetric or fluorescent assay for homogentisate or *p*-hydroxyphenylpyruvate would also be able to be readily adapted into a high capacity screen for inhibitors of this enzyme. The isolated overexpressed enzyme has sufficient activity to be used directly in a spectrophotometric assay or it can be further purified for enhanced assay sensitivity.

EXAMPLE 5

Re-construction of the Full-length *p*-Hydroxyphenylpyruvate Dioxygenase Gene for Production of Active, Stable Enzyme in Bacteria

The plasmid pT7BlueR+PDO2, described in Example 2 and containing the full-length *p*-hydroxyphenylpyruvate dioxygenase gene, proved to have incorrect sequence at the EcoRI site. This was re-sequenced so that an oligonucleotide could be designed to replace the EcoRI site with an NdeI site using conventional loop-out mutagenesis. The oligonucleotide was designed so that this procedure also introduced an ATG initiation codon at the 5'- end of the *p*-hydroxyphenylpyruvate dioxygenase gene followed by the full-length *p*-hydroxyphenylpyruvate dioxygenase sequence. After mutagenesis, the clone was amplified in *E. coli* and the plasmid was purified. The resulting full-length gene, "PDO-B", was then digested with the enzymes using NdeI and NheI, and the ~820 bp fragment used to replace the NdeI - Nhe I segment of the truncated *p*-hydroxyphenylpyruvate dioxygenase gene, "PDO-A," in pE24CP1 (Example 1). The resulting plasmid, pE24PDO-B can be expressed in bacteria to produce the full-length *Arabidopsis* *p*-hydroxyphenylpyruvate dioxygenase enzyme as determined by enzyme activity and N-terminal sequence analysis.

EXAMPLE 6

Enhanced Stability of Full Length Construct Over the Truncated Construct

Two different constructs for *Arabidopsis thaliana* *p*-hydroxyphenylpyruvate dioxygenase, one containing the full-length sequence, PDO-B as described in Example 5 and produced from plasmid pE24PDO-B, and one containing the truncated sequence lacking the putative chloroplast leader sequence, PDO-A as produced from plasmid pE24CP1, were both purified to the same extent using a Pharmacia phenyl Sepharose hydrophobic interaction column followed by gel filtration chromatography on Pharmacia Sephacryl 300. The two proteins were diluted to 1 mg/mL in 20 mM bis tris-propane buffer, pH 7.2 containing 5 mM ascorbate, 1 mM reduced glutathione and 0.1 mM ferrous ammonium sulfate and stored in a refrigerator at 4 °C for up to 10 days. Aliquots were removed at various times and assayed for activity using the tautomerase

coupled spectrophotometric assay. Under these conditions the half-life for the activity of the full length enzyme was 4 days, whereas the truncated enzyme preparation had a half-life of 9 to 10 hours. In addition, the activity of the full length enzyme could be restored by incubation with iron and reducing agent, reduced glutathione or ascorbate, or by dialysis against buffer containing iron and reducing agent. In contrast, the activity of the truncated enzyme could not be restored by incubation with or dialysis against buffer containing iron and reducing agent. The full-length enzyme was also more stable in the spectrophotometric assay showing a 2 to 3 times longer useful linear region than the truncated enzyme. Both enzyme preparations showed similar I_{50} values with the herbicidally active inhibitors.

These results clearly show that the full-length PDO-B construct has decided advantages over the truncated enzyme due to the enhanced stability under storage conditions, in the spectrophotometric assay and in the reversible reconstitution of activity in the presence of iron and reducing agent. While both enzyme constructs can be used for screening of inhibitors, the PDO-B enzyme is preferred for this application and is far superior for mechanistic and structural studies.

EXAMPLE 7

Cloning of the Maize *p*-Hydroxyphenylpyruvate Dioxygenase Gene

Approximately 600,000 plaques of a Stratagene maize Uni-Zap cDNA library (from young plants) were screened by filter hybridization under moderate stringency using a heterologous probe. The probe was prepared by PCR and was a 916 bp fragment of DNA having the sequence defined by the region extending from position 263 to 1178 of SEQ ID NO:14. Twenty-four positive phage clones were identified in the primary screen, and eleven phage clones were recovered from a secondary screen. Seven positive clones were submitted for sequencing, and four showed significant conservation sequence at the amino acid level when compared with the *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase protein. The longest of the four contained an insert of 988 bp and showed 70% identity and 78% similarity with the *Arabidopsis* protein, but was lacking approximately 550 bp corresponding to the amino terminal end of the protein.

Attempts to obtain a full-length cDNA of the maize *p*-hydroxyphenylpyruvate dioxygenase gene were unsuccessful, possibly because the secondary structure of the RNA inhibited efficient reverse transcription of this transcript. Two additional cDNA libraries were screened and clones long enough to contain a full-length cDNA were sequenced. All of these clones were shown to be chimeras. Therefore a genomic library was screened to obtain the 5' one-third of

the gene. Approximately 1 million clones from a Clontech *Zea mays* (var. B73) library in the phage vector EMBL3 (whole seedlings, 2 leaf stage) were screened using a 415 bp EcoRI-BssHII fragment containing the 5' end of the truncated corn *p*-hydroxyphenylpyruvate dioxygenase cDNA (clone H1011C). Eight positive primary phage clones were plated and screened, and four secondary clones were picked. DNA was prepared from each using the Qiagen Lambda midi-kit. Restriction digests with SalI or EcoRI indicated that two clones were the same. DNA samples from the remaining 3 clones (11.1.3, 13.1.1, and 21.2.1) were digested with SalI, EcoRI, or SalI and EcoRI, prepared for Southern analysis, and probed with the full length *Arabidopsis p*-hydroxyphenylpyruvate dioxygenase gene. Two of the clones (11.1.3 and 13.1.1) showed sequence conservation, and these homologous fragments were subcloned and sequenced. Both clones appeared to contain the full-length gene and each contained one intron near the 3' end of the gene. However, there were differences between the sequences of the two clones indicating that they may be two different genes or one may be a pseudogene. The sequence of clone 11.1.3 matched the cDNA sequence, and this clone was used to construct a full length *p*-hydroxyphenylpyruvate dioxygenase coding region.

The gene was contained on two adjacent fragments, a 3.5 kb EcoRI - SalI fragment and a 2 kb SalI fragment. Both were subcloned into pBluescript SKII+ resulting in the plasmids pES1113 and pSal1113. pES1113 was digested with SpeI to release approximately 2.7 kb of upstream sequence and then religated, resulting in a plasmid with an insert of 747 base pairs (pSPE1). pSPE1 was digested with SalI to linearize the plasmid and ligated with the 2 kb SalI fragment from pSal1113, which had been released by digestion with SalI and gel purified. Orientation was confirmed by digestion with SpeI and Bpu1102I and the correct plasmid was named p1113. In order to remove the intron contained in the 3' end of the genomic clone, the plasmid was digested with Bpu1102I and XhoI and the 3.9 kb fragment containing the vector and 5' part of the gene was gel purified. The corresponding 882 bp Bpu1102I-XhoI fragment from pH1011c (cDNA)was gel purified and ligated with this 3.9 kb fragment resulting in the clone pMPDO (ATCC 209120), which contains a 1782 bp insert. There are 260 base pairs upstream of the putative ATG and 189 base pairs downstream of the stop codon. The full-length sequence was confirmed by sequencing across the insert. The nucleic acid sequence and the deduced protein sequence for corn *p*-hydroxyphenylpyruvate dioxygenase are presented in SEQ ID NOS:10 and 11, respectively. The sequences for *p*-hydroxyphenylpyruvate dioxygenases obtained from corn and *Arabidopsis* were compared using the "Gap" program of GCG

(Program Manual for the Wisconsin Package, Version 9.0-OpenVMS, December 1996, Genetics Computer Group, 575 Science Drive, Madison, WI, USA 53711). The results of these comparisons indicated that these functions are approximately 67% identical at the nucleotide level, and they possess 69% similarity and 62% identity at the amino acid level. The predicted amino acid sequence of corn *p*-hydroxyphenylpyruvate dioxygenase is compared with that from *Arabidopsis* and other eukaryotes in Figure 3.

EXAMPLE 8

Composition of a cDNA Library; Isolation and Sequencing of cDNA Clones

A cDNA library representing mRNAs from developing seeds of *Vernonia galamenensis* that had just begun production of vernolic acid was prepared. The library was prepared in a Uni-ZAP™ XR vector according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). Conversion of the Uni-ZAP™ XR library into a plasmid library was accomplished according to the protocol provided by Stratagene. Upon conversion, cDNA inserts were contained in the plasmid vector pBluescript. cDNA inserts from randomly picked bacterial colonies containing recombinant pBluescript plasmids were amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Amplified insert DNAs were sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"; see Adams, M. D. et al., (1991) *Science 252*:1651). The resulting ESTs were analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

EXAMPLE 9

Identification and Characterization of cDNA Clones

ESTs encoding *Vernonia galamenensis* enzymes were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul, S. F. et al., (1993) *J. Mol. Biol.* 215:403-410; see also www.ncbi.nlm.nih.gov/BLAST/) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences obtained in Example 9 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish, W. and States, D. J.

(1993) *Nature Genetics 3*:266-272) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

The BLASTX search using clone vs1.pk0015.b2 revealed similarity of the protein encoded by the cDNA to a number of *p*-hydroxyphenylpyruvate dioxygenases from sources other that plants. The three most similar *p*-hydroxyphenylpyruvate dioxygenase proteins were a streptomycete *p*-hydroxyphenylpyruvate dioxygenase (GenBank Accession No. U11864; pLog = 8.34), a rat *p*-hydroxyphenylpyruvate dioxygenase (GenBank Accession No. M18405; pLog = 7.66), and a human *p*-hydroxyphenylpyruvate dioxygenase (GenBank Accession No. U29895; pLog = 7.60). SEQ ID NO:16 shows the nucleotide sequence of a portion of the *Vernonia galamenensis* cDNA in clone vs1.pk0015.b2. Sequence alignments and BLAST scores and probabilities indicate that the instant nucleic acid fragment encodes a portion of *Vernonia galamenensis* *p*-hydroxyphenylpyruvate dioxygenase.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: E. I. DUPONT DE NEMOURS AND COMPANY
(B) STREET: 1007 MARKET STREET
(C) CITY: WILMINGTON
(D) STATE: DELAWARE
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 19898
(G) TELEPHONE: 302-892-8112
(H) TELEFAX: 302-773-0164
(I) TELEX: 6717325

(ii) TITLE OF INVENTION: PLANT GENE FOR *p*-HYDROXY-PHENYLPYRUVATE DIOXYGENASE

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE, 3.50 INCH
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: MICROSOFT WORD FOR WINDOWS 95
(D) SOFTWARE: MICROSOFT WORD VERSION 7.0A

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/021,364
(B) FILING DATE: JUNE 27, 1996

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: FLOYD, LINDA AXAMETHY
(B) REGISTRATION NUMBER: 33,692
(C) REFERENCE/DOCKET NUMBER: BA-9120

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CAAGAAACGN GTCGNCGACG TGCTCAGCGA TGATCAGATC AAGGAGTGTG AGGAATTAGG   60

GATTCTTNTA GACAGAGATG ATCAAGGGAC GTTNCTTCAA ATCTNCACAA AACCACTAGG  120

TGACAGGCCG ACGNTATTTA TAGAGATAAT CCAGAGNGTA GGATGCATGA TGAAAGATGT  180

GGAAGGGANG GCTTACCAGA GTGGAGNATN TNGTGGTTTT GGCAAAGGCA ATT          233
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1448 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 9..1343

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
TGAAATCA ATG GGC CAC CAA AAC GCC GCC GTT TCA GAG AAT CAA AAC CAT      50
         Met Gly His Gln Asn Ala Ala Val Ser Glu Asn Gln Asn His
           1               5                  10

GAT GAC GGC GCT GCG TCG TCG CCG GGA TTC AAG CTC GTC GGA TTT TCC       98
Asp Asp Gly Ala Ala Ser Ser Pro Gly Phe Lys Leu Val Gly Phe Ser
 15                  20                  25                  30

AAG TTC GTA AGA AAG AAT CCA AAG TCT GAT AAA TTC AAG GTT AAG CGC      146
Lys Phe Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val Lys Arg
                 35                  40                  45

TTC CAT CAC ATC GAG TTC TGG TGC GGG GAC GCA ACC AAC GTC GCT CGT      194
Phe His His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val Ala Arg
             50                  55                  60

CGC TTC TCC TGG GGT CTG GGG ATG AGA TTC TCC GCC AAA TCC GAT CTT      242
Arg Phe Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser Asp Leu
         65                  70                  75

TCC ACC GGA AAC ATG GTT CAC GCC TCT TAC CTA CTC ACC TCC GGT GAA      290
Ser Thr Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser Gly Glu
     80                  85                  90

CTC CGA TTC CTT TTC ACT GCT CCT TAC TCT CCG TCT CTC TCC GGC GGA      338
Leu Arg Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser Gly Gly
 95                 100                 105                 110
```

```
GAG ATT AAA CCG ACA ACC ACA GGT TCT ATC CCA AGT TTC GAT CAC GGG     386
Glu Ile Lys Pro Thr Thr Thr Gly Ser Ile Pro Ser Phe Asp His Gly
                115                 120                 125

TCT TGT CGG TCC TTC TTC TCT TCA CAT GGT CTC GGT GTT AGA CCC GTT     434
Ser Cys Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg Pro Val
            130                 135                 140

GCG ATT GAA GTA GAA GAC GCG GAG TCA GCT TTC TCC ATC AGT GTA GCT     482
Ala Ile Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser Val Ala
        145                 150                 155

AAT GGC GCT ATT CCT TCG TCG CCT CCT ATC GTC CTC AAT GAA GCA GTT     530
Asn Gly Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu Ala Val
    160                 165                 170

ACG ATC GCT GAG GTT AAA CTA TAC GGC GAT GTT GTT CTC CGA TAT GTT     578
Thr Ile Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg Tyr Val
175                 180                 185                 190

AGT TAC AAA GCA GAA GAT ACC GAA AAA TCC GAA TTC TTG CCA GGG TTC     626
Ser Tyr Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro Gly Phe
                195                 200                 205

GAG CGT GTA GAG GAT GCG TCG TCG TTC CCA TTG GAT TAT GGT ATC CGG     674
Glu Arg Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly Ile Arg
            210                 215                 220

CGG CTT GAC CAC GCC GTG GGA AAC GTT CCT GAG CTT GGT CCG GCT TTA     722
Arg Leu Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro Ala Leu
        225                 230                 235

ACT TAT GTA GCG GGG TTC ACT GGT TTT CAC CAA TTC GCA GAG TTC ACA     770
Thr Tyr Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu Phe Thr
    240                 245                 250

GCA GAC GAC GTT GGA ACC GCC GAG AGC GGT TTA AAT TCA GCG GTC CTG     818
Ala Asp Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala Val Leu
255                 260                 265                 270

GCT AGC AAT GAT GAA ATG GTT CTT CTA CCG ATT AAC GAG CCA GTG CAC     866
Ala Ser Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro Val His
                275                 280                 285

GGA ACA AAG AGG AAG AGT CAG ATT CAG ACG TAT TTG GAA CAT AAC GAA     914
Gly Thr Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His Asn Glu
            290                 295                 300

GGC GCA GGG CTA CAA CAT CTG GCT CTG ATG AGT GAA GAC ATA TTC AGG     962
Gly Ala Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile Phe Arg
        305                 310                 315

ACC CTG AGA GAG ATG AGG AAG AGG AGC AGT ATT GGA GGA TTC GAC TTC    1010
Thr Leu Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe Asp Phe
    320                 325                 330

ATG CCT TCT CCT CCG CCT ACT TAC TAC CAG AAT CTC AAG AAA CGG GTC    1058
Met Pro Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys Arg Val
335                 340                 345                 350

GGC GAC GTG CTC AGC GAT GAT CAG ATC AAG GAG TGT GAG GAA TTA GGG    1106
Gly Asp Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu Leu Gly
                355                 360                 365
```

ATT CTT GTA GAC AGA GAT GAT CAA GGG ACG TTG CTT CAA ATC TTC ACA 1154
Ile Leu Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile Phe Thr
370 375 380

AAA CCA CTA GGT GAC AGG CCG ACG ATA TTT ATA GAG ATA ATC CAG AGA 1202
Lys Pro Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu Ile Ile Gln Arg
385 390 395

GTA GGA TGC ATG ATG AAA GAT GAG GAA GGG AAG GCT TAC CAG AGT GGA 1250
Val Gly Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln Ser Gly
400 405 410

GGA TGT GGT GGT TTT GCC AAA GGC AAT TTC TCT GAG CTC TTC AAG TCC 1298
Gly Cys Gly Gly Phe Ala Lys Gly Asn Phe Ser Glu Leu Phe Lys Ser
415 420 425 430

ATT GAA GAA TAC GAA AAG ACT CTT GAA GCC AAA CAG TTA GTG GGA 1343
Ile Glu Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val Gly
435 440 445

TGAACAAGAA GAAGAACCAA CTAAAGGATT GTGTAATTAA TGTAAAACTG TTTTATCTTA 1403

TCAAAACAAT GTATACAACA TCTCATTTAA AAACGAGATC AATCC 1448

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 445 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Gly His Gln Asn Ala Ala Val Ser Glu Asn Gln Asn His Asp Asp
1 5 10 15

Gly Ala Ala Ser Ser Pro Gly Phe Lys Leu Val Gly Phe Ser Lys Phe
20 25 30

Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val Lys Arg Phe His
35 40 45

His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val Ala Arg Arg Phe
50 55 60

Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser Asp Leu Ser Thr
65 70 75 80

Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser Gly Glu Leu Arg
85 90 95

Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser Gly Gly Glu Ile
100 105 110

Lys Pro Thr Thr Thr Gly Ser Ile Pro Ser Phe Asp His Gly Ser Cys
115 120 125

Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg Pro Val Ala Ile
130 135 140

Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser Val Ala Asn Gly
145 150 155 160

```
Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu Ala Val Thr Ile
                165                 170                 175

Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg Tyr Val Ser Tyr
            180                 185                 190

Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro Gly Phe Glu Arg
        195                 200                 205

Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly Ile Arg Arg Leu
    210                 215                 220

Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro Ala Leu Thr Tyr
225                 230                 235                 240

Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu Phe Thr Ala Asp
                245                 250                 255

Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala Val Leu Ala Ser
            260                 265                 270

Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro Val His Gly Thr
        275                 280                 285

Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His Asn Glu Gly Ala
    290                 295                 300

Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile Phe Arg Thr Leu
305                 310                 315                 320

Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe Asp Phe Met Pro
                325                 330                 335

Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys Arg Val Gly Asp
            340                 345                 350

Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu Leu Gly Ile Leu
        355                 360                 365

Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile Phe Thr Lys Pro
    370                 375                 380

Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu Ile Ile Gln Arg Val Gly
385                 390                 395                 400

Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln Ser Gly Gly Cys
                405                 410                 415

Gly Gly Phe Ala Lys Gly Asn Phe Ser Glu Leu Phe Lys Ser Ile Glu
            420                 425                 430

Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val Gly
        435                 440                 445
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 53 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATGTCCAAG TTCGTAAGAA AGAATCCAAA GTCTGATAAA TTCAAGGTTA AGC 53

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTTAACCTT GAATTTATCA GACTTTGGAT TCTTTCTTAC GAACTTGGAC A 51

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 392 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Thr Ser Tyr Ser Asp Lys Gly Glu Lys Pro Glu Arg Gly Arg Phe Leu
1               5                   10                  15

His Phe His Ser Val Thr Phe Trp Val Gly Asn Ala Lys Gln Ala Ala
            20                  25                  30

Ser Tyr Tyr Cys Ser Lys Ile Gly Phe Glu Pro Leu Ala Tyr Lys Gly
        35                  40                  45

Leu Glu Thr Gly Ser Arg Glu Val Val Ser His Val Val Lys Gln Asp
    50                  55                  60

Lys Ile Val Phe Val Phe Ser Ser Ala Leu Asn Pro Trp Asn Lys Glu
65                  70                  75                  80

Met Gly Asp His Leu Val Lys His Gly Asp Gly Val Lys Asp Ile Ala
                85                  90                  95

Phe Glu Val Glu Asp Cys Asp Tyr Ile Val Gln Lys Ala Arg Glu Arg
            100                 105                 110

Gly Ala Ile Ile Val Arg Glu Glu Val Cys Cys Ala Ala Asp Val Arg
        115                 120                 125

Gly His His Thr Pro Leu Asp Arg Ala Arg Gln Val Trp Glu Gly Thr
    130                 135                 140

Leu Val Glu Lys Met Thr Phe Cys Leu Asp Ser Arg Pro Gln Pro Ser
145                 150                 155                 160

Gln Thr Leu Leu His Arg Leu Leu Leu Ser Lys Leu Pro Lys Cys Gly
                165                 170                 175

Leu Glu Ile Ile Asp His Ile Val Gly Asn Gln Pro Asp Gln Glu Met
            180                 185                 190
```

```
Glu Ser Ala Ser Gln Trp Tyr Met Arg Asn Leu Gln Phe His Arg Phe
        195                 200                 205

Trp Ser Val Asp Asp Thr Gln Ile His Thr Glu Tyr Ser Ala Leu Arg
    210                 215                 220

Ser Val Val Met Ala Asn Tyr Glu Glu Ser Ile Lys Met Pro Ile Asn
225                 230                 235                 240

Glu Pro Ala Pro Gly Lys Lys Lys Ser Gln Ile Gln Glu Tyr Val Asp
                245                 250                 255

Tyr Asn Gly Gly Ala Gly Val Gln His Ile Ala Leu Lys Thr Glu Asp
            260                 265                 270

Ile Ile Thr Ala Ile Arg Ser Leu Arg Glu Arg Gly Val Glu Phe Leu
        275                 280                 285

Ala Val Pro Phe Thr Tyr Tyr Lys Gln Leu Gln Glu Lys Leu Lys Ser
    290                 295                 300

Ala Lys Ile Arg Val Lys Glu Ser Ile Asp Val Leu Glu Glu Leu Lys
305                 310                 315                 320

Ile Leu Val Asp Tyr Asp Glu Lys Gly Tyr Leu Leu Gln Ile Phe Thr
                325                 330                 335

Lys Pro Met Gln Asp Arg Pro Thr Val Phe Leu Glu Val Ile Gln Arg
            340                 345                 350

Asn Asn His Gln Gly Phe Gly Ala Gly Asn Phe Asn Ser Leu Phe Lys
        355                 360                 365

Ala Phe Glu Glu Glu Gln Glu Leu Arg Gly Asn Leu Thr Asp Thr Asp
    370                 375                 380

Pro Asn Gly Val Pro Phe Arg Leu
385                 390
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 392 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Thr Ser Tyr Ser Asp Lys Gly Glu Lys Pro Glu Arg Gly Arg Phe Leu
1               5                   10                  15

His Phe His Ser Val Thr Phe Trp Val Gly Asn Ala Lys Gln Ala Ala
            20                  25                  30

Ser Tyr Tyr Cys Ser Lys Ile Gly Phe Glu Pro Leu Ala Tyr Lys Gly
        35                  40                  45

Leu Glu Thr Gly Ser Arg Glu Val Val Ser His Val Val Lys Gln Asp
    50                  55                  60

Lys Ile Val Phe Val Phe Ser Ser Ala Leu Asn Pro Trp Asn Lys Glu
65                  70                  75                  80
```

Met Gly Asp His Leu Val Lys His Gly Asp Gly Val Lys Asp Ile Ala
85 90 95

Phe Glu Val Glu Asp Cys Asp Tyr Ile Val Gln Lys Ala Arg Glu Arg
100 105 110

Gly Ala Ile Ile Val Arg Glu Glu Val Cys Cys Ala Ala Asp Val Arg
115 120 125

Gly His His Thr Pro Leu Asp Arg Ala Arg Gln Val Trp Glu Gly Thr
130 135 140

Leu Val Glu Lys Met Thr Phe Cys Leu Asp Ser Arg Pro Gln Pro Ser
145 150 155 160

Gln Thr Leu Leu His Arg Leu Leu Leu Ser Lys Leu Pro Lys Cys Gly
165 170 175

Leu Glu Ile Ile Asp His Ile Val Gly Asn Gln Pro Asp Gln Glu Met
180 185 190

Glu Ser Ala Ser Gln Trp Tyr Met Arg Asn Leu Gln Phe His Arg Phe
195 200 205

Trp Ser Val Asp Asp Thr Gln Ile His Thr Glu Tyr Ser Ala Leu Arg
210 215 220

Ser Val Val Met Ala Asn Tyr Glu Glu Ser Ile Lys Met Pro Ile Asn
225 230 235 240

Glu Pro Ala Pro Gly Lys Lys Lys Ser Gln Ile Gln Glu Tyr Val Asp
245 250 255

Tyr Asn Gly Gly Ala Gly Val Gln His Ile Ala Leu Lys Thr Glu Asp
260 265 270

Ile Ile Thr Ala Ile Arg Ser Leu Arg Glu Arg Gly Val Glu Phe Leu
275 280 285

Ala Val Pro Phe Thr Tyr Tyr Lys Gln Leu Gln Glu Lys Leu Lys Ser
290 295 300

Ala Lys Ile Arg Val Lys Glu Ser Ile Asp Val Leu Glu Glu Leu Lys
305 310 315 320

Ile Leu Val Asp Tyr Asp Glu Lys Gly Tyr Leu Leu Gln Ile Phe Thr
325 330 335

Lys Pro Met Gln Asp Arg Pro Thr Val Phe Leu Glu Val Ile Gln Arg
340 345 350

Asn Asn His Gln Gly Phe Gly Ala Gly Asn Phe Asn Ser Leu Phe Lys
355 360 365

Ala Phe Glu Glu Glu Gln Glu Leu Arg Gly Asn Leu Thr Asp Thr Asp
370 375 380

Pro Asn Gly Val Pro Phe Arg Leu
385 390

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 392 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Thr Thr Tyr Asn Asn Lys Gly Pro Lys Pro Glu Arg Gly Arg Phe Leu
1               5                   10                  15

His Phe His Ser Val Thr Phe Trp Val Gly Asn Ala Lys Gln Ala Ala
            20                  25                  30

Ser Phe Tyr Cys Asn Lys Met Gly Phe Glu Pro Leu Ala Tyr Arg Gly
        35                  40                  45

Leu Glu Thr Gly Ser Arg Glu Val Val Ser His Val Ile Lys Arg Gly
    50                  55                  60

Lys Ile Val Phe Val Leu Cys Ser Ala Leu Asn Pro Trp Asn Lys Glu
65                  70                  75                  80

Met Gly Asp His Leu Val Lys His Gly Asp Gly Val Lys Asp Ile Ala
                85                  90                  95

Phe Glu Val Glu Asp Cys Asp His Ile Val Gln Lys Ala Arg Glu Arg
            100                 105                 110

Gly Ala Lys Ile Val Arg Glu Pro Trp Val Glu Gln Asp Lys Phe Gly
        115                 120                 125

Lys Val Lys Phe Ala Val Leu Gln Thr Tyr Gly Asp Thr Thr His Thr
    130                 135                 140

Leu Val Glu Lys Ile Asn Tyr Thr Gly Arg Phe Leu Pro Gly Phe Glu
145                 150                 155                 160

Ala Pro Thr Tyr Lys Asp Thr Leu Leu Pro Lys Leu Pro Arg Cys Asn
                165                 170                 175

Leu Glu Ile Ile Asp His Ile Val Gly Asn Gln Pro Asp Gln Glu Met
            180                 185                 190

Gln Ser Ala Ser Glu Trp Tyr Leu Lys Asn Leu Gln Phe His Arg Phe
        195                 200                 205

Trp Ser Val Asp Asp Thr Gln Val His Thr Glu Tyr Ser Ser Leu Arg
    210                 215                 220

Ser Ile Val Val Thr Asn Tyr Glu Glu Ser Ile Lys Met Pro Ile Asn
225                 230                 235                 240

Glu Pro Ala Pro Gly Arg Lys Lys Ser Gln Ile Gln Glu Tyr Val Asp
                245                 250                 255

Tyr Asn Gly Gly Ala Gly Val Gln His Ile Ala Leu Lys Thr Glu Asp
            260                 265                 270

Ile Ile Thr Ala Ile Arg His Leu Arg Glu Arg Gly Thr Glu Phe Leu
        275                 280                 285

Ala Ala Pro Ser Ser Tyr Tyr Lys Leu Leu Arg Glu Asn Leu Lys Ser
    290                 295                 300

Ala Lys Ile Gln Val Lys Glu Ser Met Asp Val Leu Glu Glu Leu His
305                 310                 315                 320
```

Ile Leu Val Asp Tyr Asp Glu Lys Gly Tyr Leu Leu Gln Ile Phe Thr
325 330 335

Lys Pro Met Gln Asp Arg Pro Thr Leu Phe Leu Glu Val Ile Gln Arg
340 345 350

His Asn His Gln Gly Phe Gly Ala Gly Asn Phe Asn Ser Leu Phe Lys
355 360 365

Ala Phe Glu Glu Glu Gln Ala Leu Arg Gly Asn Leu Thr Asp Leu Glu
370 375 380

Pro Asn Gly Val Arg Ser Gly Met
385 390

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 376 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Tyr Trp Asp Lys Gly Pro Lys Pro Glu Arg Gly Arg Phe Leu His Phe
1 5 10 15

His Ser Val Thr Phe Trp Val Gly Asn Ala Lys Gln Ala Ala Ser Phe
20 25 30

Tyr Cys Asn Lys Met Gly Phe Glu Pro Leu Ala Tyr Lys Gly Leu Glu
35 40 45

Thr Gly Ser Arg Glu Val Val Ser His Val Ile Lys Gln Gly Lys Ile
50 55 60

Val Phe Val Leu Cys Ser Ala Leu Asn Pro Trp Asn Lys Glu Met Gly
65 70 75 80

Asp His Leu Val Lys His Gly Asp Gly Val Lys Asp Ile Ala Phe Glu
85 90 95

Val Glu Asp Cys Glu His Ile Val Gln Lys Ala Arg Glu Arg Gly Ala
100 105 110

Lys Ile Val Arg Glu Pro Trp Val Glu Glu Asp Lys Phe Gly Lys Val
115 120 125

Lys Phe Ala Val Leu Gln Thr Tyr Gly Asp Thr Thr His Thr Leu Val
130 135 140

Glu Lys Ile Asn Tyr Thr Gly Arg Phe Leu Pro Gly Phe Glu Ala Pro
145 150 155 160

Thr Tyr Lys Asp Thr Leu Leu Pro Lys Leu Pro Ser Cys Asn Leu Glu
165 170 175

Ile Ile Asp His Ile Val Gly Asn Gln Pro Asp Gln Glu Met Glu Ser
180 185 190

Ala Ser Glu Trp Tyr Leu Lys Asn Leu Gln Phe His Arg Phe Trp Ser
195 200 205

```
Val Asp Asp Thr Gln Val His Thr Glu Tyr Ser Ser Leu Arg Ser Ile
    210                 215                 220

Val Val Ala Asn Tyr Glu Glu Ser Ile Lys Met Pro Ile Asn Glu Pro
225                 230                 235                     240

Ala Pro Gly Arg Lys Lys Ser Gln Ile Gln Glu Tyr Val Asp Tyr Asn
                245                 250                     255

Gly Gly Ala Gly Val Gln His Ile Ala Leu Arg Thr Glu Asp Ile Ile
            260                 265                     270

Thr Thr Ile Arg His Leu Arg Glu Arg Gly Met Glu Phe Leu Ala Val
        275                 280                     285

Pro Ser Ser Tyr Tyr Arg Leu Leu Arg Glu Asn Leu Lys Thr Ser Lys
    290                 295                     300

Ile Gln Val Lys Glu Asn Met Asp Val Leu Glu Glu Leu Lys Ile Leu
305                 310                 315                     320

Val Asp Tyr Asp Glu Lys Gly Tyr Leu Leu Gln Ile Phe Thr Lys Pro
                325                 330                     335

Met Gln Asp Arg Pro Thr Leu Phe Leu Glu Val Ile Gln Arg His Asn
            340                 345                     350

His Gln Gly Phe Gly Ala Gly Asn Phe Asn Ser Leu Phe Lys Ala Phe
        355                 360                     365

Glu Glu Glu Gln Ala Leu Arg Gly
    370                 375
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1766 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Zea mays

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 261..1595

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
ACTAGTTGTG AGAGCCTTCT GCGTTGGCAA TTGGCAGTAC AAGACAAATC ACATCCGCAA    60

CCGCAACCAC AGAATCGTCC GTCCACGTGG CCCCCATCAC TTCCCTTTAT TTACCAGTCG   120

TCCCCCATCC CCAGGGCCAC CCACCAACAA GTGCAGTCAC CCGAGCCGCA AACTGCAGCT   180

CTGCAAGCTA CAGAGGCCAC CACGAGTCCA CGACGCCACG CCCTCCGAGA GAAAGAGAAA   240
```

```
GAGAAAACCA AAGCACGATA ATG CCC CCG ACC CCC ACA GCC GCC GCA GCC      290
                      Met Pro Pro Thr Pro Thr Ala Ala Ala Ala
                       1               5                  10

GGC GCC GCC GTG GCG GCG GCA TCA GCA GCG GAG CAA GCG GCG TTC CGC    338
Gly Ala Ala Val Ala Ala Ala Ser Ala Ala Glu Gln Ala Ala Phe Arg
                 15                  20                  25

CTC GTG GGC CAC CGC AAC TTC GTC CGC TTC AAC CCG CGC TCC GAC CGC    386
Leu Val Gly His Arg Asn Phe Val Arg Phe Asn Pro Arg Ser Asp Arg
             30                  35                  40

TTC CAC ACG CTC GCG TTC CAC CAC GTG GAG CTC TGG TGC GCC GAC GCG    434
Phe His Thr Leu Ala Phe His His Val Glu Leu Trp Cys Ala Asp Ala
         45                  50                  55

GCC TCC GCC GCG GGC CGC TTC TCC TTC GGC CTG GGC GCG CCG CTC GCC    482
Ala Ser Ala Ala Gly Arg Phe Ser Phe Gly Leu Gly Ala Pro Leu Ala
     60                  65                  70

GCA CGC TCC GAC CTC TCC ACG GGC AAC TCC GCG CAC GCG TCC CTG CTG    530
Ala Arg Ser Asp Leu Ser Thr Gly Asn Ser Ala His Ala Ser Leu Leu
 75                  80                  85                  90

CTC CGC TCC GGC TCC CTC TCC TTC CTC TTC ACG GCG CCC TAC GCG CAC    578
Leu Arg Ser Gly Ser Leu Ser Phe Leu Phe Thr Ala Pro Tyr Ala His
                 95                 100                 105

GGC GCC GAC GCT GCC ACC GCC GCG CTG CCC TCC TTC TCC GCC GCC GCC    626
Gly Ala Asp Ala Ala Thr Ala Ala Leu Pro Ser Phe Ser Ala Ala Ala
            110                 115                 120

GCG CGG CGC TTC GCA GCC GAC CAC GGC CTC GCG GTG CGC GCC GTC GCG    674
Ala Arg Arg Phe Ala Ala Asp His Gly Leu Ala Val Arg Ala Val Ala
        125                 130                 135

CTC CGC GTC GCC GAC GCC GAG GAC GCC TTC CGC GCC AGC GTC GCG GCC    722
Leu Arg Val Ala Asp Ala Glu Asp Ala Phe Arg Ala Ser Val Ala Ala
    140                 145                 150

GGG GCG CGC CCG GCG TTC GGC CCC GTC GAC CTC GGC CGC GGC TTC CGC    770
Gly Ala Arg Pro Ala Phe Gly Pro Val Asp Leu Gly Arg Gly Phe Arg
155                 160                 165                 170

CTC GCC GAG GTC GAG CTC TAC GGC GAC GTC GTG CTC CGG TAC GTG AGC    818
Leu Ala Glu Val Glu Leu Tyr Gly Asp Val Val Leu Arg Tyr Val Ser
                175                 180                 185

TAC CCG GAC GGC GCC GCG GGC GAG CCC TTC CTG CCG GGG TTC GAG GGC    866
Tyr Pro Asp Gly Ala Ala Gly Glu Pro Phe Leu Pro Gly Phe Glu Gly
            190                 195                 200

GTG GCC AGC CCC GGG GCG GCC GAC TAC GGG CTG AGC AGG TTC GAC CAC    914
Val Ala Ser Pro Gly Ala Ala Asp Tyr Gly Leu Ser Arg Phe Asp His
        205                 210                 215

ATC GTC GGC AAC GTG CCG GAG CTG GCG CCC GCC GCC GCC TAC TTC GCC    962
Ile Val Gly Asn Val Pro Glu Leu Ala Pro Ala Ala Ala Tyr Phe Ala
    220                 225                 230

GGC TTC ACG GGG TTC CAC GAG TTC GCC GAG TTC ACG ACG GAG GAC GTG   1010
Gly Phe Thr Gly Phe His Glu Phe Ala Glu Phe Thr Thr Glu Asp Val
235                 240                 245                 250
```

GGC ACC GCG GAG AGC GGC CTC AAC TCC ATG GTG CTC GCC AAC AAC TCG 1058
Gly Thr Ala Glu Ser Gly Leu Asn Ser Met Val Leu Ala Asn Asn Ser
255 260 265

GAG AAC GTG CTG CTC CCG CTC AAC GAG CCG GTG CAC GGC ACC AAG CGC 1106
Glu Asn Val Leu Leu Pro Leu Asn Glu Pro Val His Gly Thr Lys Arg
270 275 280

CGC AGC CAG ATA CAA ACG TTC CTG GAC CAC CAC GGC GGC CCC GGC GTG 1154
Arg Ser Gln Ile Gln Thr Phe Leu Asp His His Gly Gly Pro Gly Val
285 290 295

CAG CAC ATG GCG CTG GCC AGC GAC GAC GTG CTC AGG ACG CTG AGG GAG 1202
Gln His Met Ala Leu Ala Ser Asp Asp Val Leu Arg Thr Leu Arg Glu
300 305 310

ATG CAG GCG CGC TCG GCC ATG GGC GGC TTC GAG TTC ATG GCG CCT CCC 1250
Met Gln Ala Arg Ser Ala Met Gly Gly Phe Glu Phe Met Ala Pro Pro
315 320 325 330

ACA TCC GAC TAC TAT GAC GGC GTG AGG CGG CGC GCC GGG GAC GTG CTC 1298
Thr Ser Asp Tyr Tyr Asp Gly Val Arg Arg Arg Ala Gly Asp Val Leu
335 340 345

ACG GAA GCA CAG ATT AAG GAG TGC CAG GAG CTA GGG GTG CTG GTG GAC 1346
Thr Glu Ala Gln Ile Lys Glu Cys Gln Glu Leu Gly Val Leu Val Asp
350 355 360

AGG GAT GAC CAG GGC GTG CTG CTC CAA ATC TTC ACC AAG CCA GTG GGG 1394
Arg Asp Asp Gln Gly Val Leu Leu Gln Ile Phe Thr Lys Pro Val Gly
365 370 375

GAC AGG CCA ACG CTG TTC TTG GAA ATC ATC CAA AGG ATC GGG TGC ATG 1442
Asp Arg Pro Thr Leu Phe Leu Glu Ile Ile Gln Arg Ile Gly Cys Met
380 385 390

GAG AAG GAT GAG AAG GGG CAA GAA TAC CAA AAG GGT GGC TGC GGC GGG 1490
Glu Lys Asp Glu Lys Gly Gln Glu Tyr Gln Lys Gly Gly Cys Gly Gly
395 400 405 410

TTC GGC AAG GGA AAC TTC TCG CAG CTG TTC AAG TCC ATC GAG GAT TAT 1538
Phe Gly Lys Gly Asn Phe Ser Gln Leu Phe Lys Ser Ile Glu Asp Tyr
415 420 425

GAG AAG TCC CTT GAA GCC AAG CAA GCT GCT GCA GCA GCT GCA GCT CAG 1586
Glu Lys Ser Leu Glu Ala Lys Gln Ala Ala Ala Ala Ala Ala Ala Gln
430 435 440

GGA TCC TAG GACAGTGCTT GGAGACGAGC AACTGCTGTG GCACTTTGTA 1635
Gly Ser

TCATGGAACA GAAATAATGA AGCGTGTTCT TTGTGACACT TGACATGCAA ATGTTTGTGT 1695

TCTGTAACCG TTGAATATAT GGGACGATGC TATGATGGTG TAATAGATGG TAGAGAGGGT 1755

ACAACCCTGA T 1766

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 445 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Pro Pro Thr Pro Thr Ala Ala Ala Ala Gly Ala Ala Val Ala Ala
1 5 10 15

Ala Ser Ala Ala Glu Gln Ala Ala Phe Arg Leu Val Gly His Arg Asn
20 25 30

Phe Val Arg Phe Asn Pro Arg Ser Asp Arg Phe His Thr Leu Ala Phe
35 40 45

His His Val Glu Leu Trp Cys Ala Asp Ala Ala Ser Ala Ala Gly Arg
50 55 60

Phe Ser Phe Gly Leu Gly Ala Pro Leu Ala Ala Arg Ser Asp Leu Ser
65 70 75 80

Thr Gly Asn Ser Ala His Ala Ser Leu Leu Leu Arg Ser Gly Ser Leu
85 90 95

Ser Phe Leu Phe Thr Ala Pro Tyr Ala His Gly Ala Asp Ala Ala Thr
100 105 110

Ala Ala Leu Pro Ser Phe Ser Ala Ala Ala Ala Arg Arg Phe Ala Ala
115 120 125

Asp His Gly Leu Ala Val Arg Ala Val Ala Leu Arg Val Ala Asp Ala
130 135 140

Glu Asp Ala Phe Arg Ala Ser Val Ala Ala Gly Ala Arg Pro Ala Phe
145 150 155 160

Gly Pro Val Asp Leu Gly Arg Gly Phe Arg Leu Ala Glu Val Glu Leu
165 170 175

Tyr Gly Asp Val Val Leu Arg Tyr Val Ser Tyr Pro Asp Gly Ala Ala
180 185 190

Gly Glu Pro Phe Leu Pro Gly Phe Glu Gly Val Ala Ser Pro Gly Ala
195 200 205

Ala Asp Tyr Gly Leu Ser Arg Phe Asp His Ile Val Gly Asn Val Pro
210 215 220

Glu Leu Ala Pro Ala Ala Ala Tyr Phe Ala Gly Phe Thr Gly Phe His
225 230 235 240

Glu Phe Ala Glu Phe Thr Thr Glu Asp Val Gly Thr Ala Glu Ser Gly
245 250 255

Leu Asn Ser Met Val Leu Ala Asn Asn Ser Glu Asn Val Leu Leu Pro
260 265 270

Leu Asn Glu Pro Val His Gly Thr Lys Arg Arg Ser Gln Ile Gln Thr
275 280 285

Phe Leu Asp His His Gly Gly Pro Gly Val Gln His Met Ala Leu Ala
290 295 300

Ser Asp Asp Val Leu Arg Thr Leu Arg Glu Met Gln Ala Arg Ser Ala
305 310 315 320

Met Gly Gly Phe Glu Phe Met Ala Pro Pro Thr Ser Asp Tyr Tyr Asp
325 330 335

```
Gly Val Arg Arg Arg Ala Gly Asp Val Leu Thr Glu Ala Gln Ile Lys
            340                 345                 350

Glu Cys Gln Glu Leu Gly Val Leu Val Asp Arg Asp Asp Gln Gly Val
        355                 360                 365

Leu Leu Gln Ile Phe Thr Lys Pro Val Gly Asp Arg Pro Thr Leu Phe
    370                 375                 380

Leu Glu Ile Ile Gln Arg Ile Gly Cys Met Glu Lys Asp Glu Lys Gly
385                 390                 395                     400

Gln Glu Tyr Gln Lys Gly Gly Cys Gly Gly Phe Gly Lys Gly Asn Phe
                405                 410                 415

Ser Gln Leu Phe Lys Ser Ile Glu Asp Tyr Glu Lys Ser Leu Glu Ala
            420                 425                 430

Lys Gln Ala Ala Ala Ala Ala Ala Ala Gln Gly Ser
        435                 440
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1356 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Arabidopsis thaliana

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1254

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..3
(D) OTHER INFORMATION: /standard_name= "translation initiation codon"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1252..1254
(D) OTHER INFORMATION: /standard_name= "translation termination codon"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
ATG TCC AAG TTC GTA AGA AAG AAT CCA AAG TCT GAT AAA TTC AAG GTT      48
Met Ser Lys Phe Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val
  1               5                  10                  15

AAG CGC TTC CAT CAC ATC GAG TTC TGG TGC GGC GAC GCA ACC AAC GTC      96
Lys Arg Phe His His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val
             20                  25                  30
```

GCT CGT CGC TTC TCC TGG GGT CTG GGG ATG AGA TTC TCC GCC AAA TCC 144
Ala Arg Arg Phe Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser
35 40 45

GAT CTT TCC ACC GGA AAC ATG GTT CAC GCC TCT TAC CTA CTC ACC TCC 192
Asp Leu Ser Thr Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser
50 55 60

GGT GAC CTC CGA TTC CTT TTC ACT GCT CCT TAC TCT CCG TCT CTC TCC 240
Gly Asp Leu Arg Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser
65 70 75 80

GCC GGA GAG ATT AAA CCG ACA ACC ACA GCT TCT ATC CCA AGT TTC GAT 288
Ala Gly Glu Ile Lys Pro Thr Thr Thr Ala Ser Ile Pro Ser Phe Asp
85 90 95

CAC GGC TCT TGT CGT TCC TTC TTC TCT TCA CAT GGT CTC GGT GTT AGA 336
His Gly Ser Cys Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg
100 105 110

GCC GTT GCG ATT GAA GTA GAA GAC GCA GAG TCA GCT TTC TCC ATC AGT 384
Ala Val Ala Ile Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser
115 120 125

GTA GCT AAT GGC GCT ATT CCT TCG TCG CCT CCT ATC GTC CTC AAT GAA 432
Val Ala Asn Gly Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu
130 135 140

GCA GTT ACG ATC GCT GAG GTT AAA CTA TAC GGC GAT GTT GTT CTC CGA 480
Ala Val Thr Ile Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg
145 150 155 160

TAT GTT AGT TAC AAA GCA GAA GAT ACC GAA AAA TCC GAA TTC TTG CCA 528
Tyr Val Ser Tyr Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro
165 170 175

GGG TTC GAG CGT GTA GAG GAT GCG TCG TCG TTC CCA TTG GAT TAT GGT 576
Gly Phe Glu Arg Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly
180 185 190

ATC CGG CGG CTT GAC CAC GCC GTG GGA AAC GTT CCT GAG CTT GGT CCG 624
Ile Arg Arg Leu Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro
195 200 205

GCT TTA ACT TAT GTA GCG GGG TTC ACT GGT TTT CAC CAA TTC GCA GAG 672
Ala Leu Thr Tyr Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu
210 215 220

TTC ACA GCA GAC GAC GTT GGA ACC GCC GAG AGC GGT TTA AAT TCA GCG 720
Phe Thr Ala Asp Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala
225 230 235 240

GTC CTG GCT AGC AAT GAT GAA ATG GTT CTT CTA CCG ATT AAC GAG CCA 768
Val Leu Ala Ser Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro
245 250 255

GTG CAC GGA ACA AAG AGG AAG AGT CAG ATT CAG ACG TAT TTG GAA CAT 816
Val His Gly Thr Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His
260 265 270

AAC GAA GGC GCA GGG CTA CAA CAT CTG GCT CTG ATG AGT GAA GAC ATA 864
Asn Glu Gly Ala Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile
275 280 285

```
TTC AGG ACC CTG AGA GAG ATG AGG AAG AGG AGC AGT ATT GGA GGA TTC      912
Phe Arg Thr Leu Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe
    290                 295                 300

GAC TTC ATG CCT TCT CCT CCG CCT ACT TAC TAC CAG AAT CTC AAG AAA      960
Asp Phe Met Pro Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys
305                 310                 315                 320

CGG GTC GGC GAC GTG CTC AGC GAT GAT CAG ATC AAG GAG TGT GAG GAA     1008
Arg Val Gly Asp Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu
                325                 330                 335

TTA GGG ATT CTT GTA GAC AGA GAT GAT CAA GGG ACG TTG CTT CAA ATC     1056
Leu Gly Ile Leu Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile
            340                 345                 350

TTC ACA AAA CCA CTA GGT GAC AGG CCG ACG ATA TTT ATA GAG ATA ATC     1104
Phe Thr Lys Pro Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu Ile Ile
        355                 360                 365

CAG AGA GTA GGA TGC ATG ATG AAA GAT GAG GAA GGG AAG GCT TAC CAG     1152
Gln Arg Val Gly Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln
    370                 375                 380

AGT GGA GGA TGT GGT GGT TTT GGC AAA GGC AAT TTC TCT GAG CTC TTC     1200
Ser Gly Gly Cys Gly Gly Phe Gly Lys Gly Asn Phe Ser Glu Leu Phe
385                 390                 395                 400

AAG TCC ATT GAA GAA TAC GAA AAG ACT CTT GAA GCC AAA CAG TTA GTG     1248
Lys Ser Ile Glu Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val
                405                 410                 415

GGA TGA ACAAGAAGAA GAACCAACTA AAGGATTGTG TAATTAATGT AAAACTGTTT      1304
Gly  *

TATCTTATCA AAACAATGTA TACAACATCT CATTTAAAAA CGAGATCAAT CC           1356
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 418 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Ser Lys Phe Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val
  1               5                  10                  15

Lys Arg Phe His His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val
             20                  25                  30

Ala Arg Arg Phe Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser
         35                  40                  45

Asp Leu Ser Thr Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser
     50                  55                  60

Gly Asp Leu Arg Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser
 65                  70                  75                  80

Ala Gly Glu Ile Lys Pro Thr Thr Thr Ala Ser Ile Pro Ser Phe Asp
                 85                  90                  95
```

His Gly Ser Cys Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg
100 105 110

Ala Val Ala Ile Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser
115 120 125

Val Ala Asn Gly Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu
130 135 140

Ala Val Thr Ile Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg
145 150 155 160

Tyr Val Ser Tyr Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro
165 170 175

Gly Phe Glu Arg Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly
180 185 190

Ile Arg Arg Leu Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro
195 200 205

Ala Leu Thr Tyr Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu
210 215 220

Phe Thr Ala Asp Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala
225 230 235 240

Val Leu Ala Ser Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro
245 250 255

Val His Gly Thr Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His
260 265 270

Asn Glu Gly Ala Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile
275 280 285

Phe Arg Thr Leu Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe
290 295 300

Asp Phe Met Pro Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys
305 310 315 320

Arg Val Gly Asp Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu
325 330 335

Leu Gly Ile Leu Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile
340 345 350

Phe Thr Lys Pro Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu Ile Ile
355 360 365

Gln Arg Val Gly Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln
370 375 380

Ser Gly Gly Cys Gly Gly Phe Gly Lys Gly Asn Phe Ser Glu Leu Phe
385 390 395 400

Lys Ser Ile Glu Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val
405 410 415

Gly

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1448 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Arabidopsis thaliana

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 9..1346

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 9..11
(D) OTHER INFORMATION: /standard_name= "translation initiation codon"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1344..1346
(D) OTHER INFORMATION: /standard_name= "translation termination codon"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
TGAAATCA ATG GGC CAC CAA AAC GCC GCC GTT TCA GAG AAT CAA AAC CAT      50
         Met Gly His Gln Asn Ala Ala Val Ser Glu Asn Gln Asn His
           1               5                  10

GAT GAC GGC GCT GCG TCG TCG CCG GGA TTC AAG CTC GTC GGA TTT TCC       98
Asp Asp Gly Ala Ala Ser Ser Pro Gly Phe Lys Leu Val Gly Phe Ser
 15                  20                  25                  30

AAG TTC GTA AGA AAG AAT CCA AAG TCT GAT AAA TTC AAG GTT AAG CGC      146
Lys Phe Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val Lys Arg
                 35                  40                  45

TTC CAT CAC ATC GAG TTC TGG TGC GGC GAC GCA ACC AAC GTC GCT CGT      194
Phe His His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val Ala Arg
             50                  55                  60

CGC TTC TCC TGG GGT CTG GGG ATG AGA TTC TCC GCC AAA TCC GAT CTT      242
Arg Phe Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser Asp Leu
         65                  70                  75

TCC ACC GGA AAC ATG GTT CAC GCC TCT TAC CTA CTC ACC TCC GGT GAC      290
Ser Thr Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser Gly Asp
     80                  85                  90

CTC CGA TTC CTT TTC ACT GCT CCT TAC TCT CCG TCT CTC TCC GCC GGA      338
Leu Arg Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser Ala Gly
 95                 100                 105                 110

GAG ATT AAA CCG ACA ACC ACA GCT TCT ATC CCA AGT TTC GAT CAC GGC      386
Glu Ile Lys Pro Thr Thr Thr Ala Ser Ile Pro Ser Phe Asp His Gly
                115                 120                 125
```

```
TCT TGT CGT TCC TTC TTC TCT TCA CAT GGT CTC GGT GTT AGA GCC GTT     434
Ser Cys Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg Ala Val
            130                 135                 140

GCG ATT GAA GTA GAA GAC GCA GAG TCA GCT TTC TCC ATC AGT GTA GCT     482
Ala Ile Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser Val Ala
        145                 150                 155

AAT GGC GCT ATT CCT TCG TCG CCT CCT ATC GTC CTC AAT GAA GCA GTT     530
Asn Gly Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu Ala Val
    160                 165                 170

ACG ATC GCT GAG GTT AAA CTA TAC GGC GAT GTT GTT CTC CGA TAT GTT     578
Thr Ile Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg Tyr Val
175                 180                 185                 190

AGT TAC AAA GCA GAA GAT ACC GAA AAA TCC GAA TTC TTG CCA GGG TTC     626
Ser Tyr Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro Gly Phe
                195                 200                 205

GAG CGT GTA GAG GAT GCG TCG TCG TTC CCA TTG GAT TAT GGT ATC CGG     674
Glu Arg Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly Ile Arg
            210                 215                 220

CGG CTT GAC CAC GCC GTG GGA AAC GTT CCT GAG CTT GGT CCG GCT TTA     722
Arg Leu Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro Ala Leu
        225                 230                 235

ACT TAT GTA GCG GGG TTC ACT GGT TTT CAC CAA TTC GCA GAG TTC ACA     770
Thr Tyr Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu Phe Thr
    240                 245                 250

GCA GAC GAC GTT GGA ACC GCC GAG AGC GGT TTA AAT TCA GCG GTC CTG     818
Ala Asp Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala Val Leu
255                 260                 265                 270

GCT AGC AAT GAT GAA ATG GTT CTT CTA CCG ATT AAC GAG CCA GTG CAC     866
Ala Ser Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro Val His
                275                 280                 285

GGA ACA AAG AGG AAG AGT CAG ATT CAG ACG TAT TTG GAA CAT AAC GAA     914
Gly Thr Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His Asn Glu
            290                 295                 300

GGC GCA GGG CTA CAA CAT CTG GCT CTG ATG AGT GAA GAC ATA TTC AGG     962
Gly Ala Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile Phe Arg
        305                 310                 315

ACC CTG AGA GAG ATG AGG AAG AGG AGC AGT ATT GGA GGA TTC GAC TTC    1010
Thr Leu Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe Asp Phe
    320                 325                 330

ATG CCT TCT CCT CCG CCT ACT TAC TAC CAG AAT CTC AAG AAA CGG GTC    1058
Met Pro Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys Arg Val
335                 340                 345                 350

GGC GAC GTG CTC AGC GAT GAT CAG ATC AAG GAG TGT GAG GAA TTA GGG    1106
Gly Asp Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu Leu Gly
                355                 360                 365

ATT CTT GTA GAC AGA GAT GAT CAA GGG ACG TTG CTT CAA ATC TTC ACA    1154
Ile Leu Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile Phe Thr
            370                 375                 380
```

```
AAA CCA CTA GGT GAC AGG CCG ACG ATA TTT ATA GAG ATA ATC CAG AGA    1202
Lys Pro Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu Ile Ile Gln Arg
        385                 390                 395

GTA GGA TGC ATG ATG AAA GAT GAG GAA GGG AAG GCT TAC CAG AGT GGA    1250
Val Gly Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln Ser Gly
    400                 405                 410

GGA TGT GGT GGT TTT GGC AAA GGC AAT TTC TCT GAG CTC TTC AAG TCC    1298
Gly Cys Gly Gly Phe Gly Lys Gly Asn Phe Ser Glu Leu Phe Lys Ser
415                 420                 425                 430

ATT GAA GAA TAC GAA AAG ACT CTT GAA GCC AAA CAG TTA GTG GGA TGA    1346
Ile Glu Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val Gly  *
            435                 440                 445

ACAAGAAGAA GAACCAACTA AAGGATTGTG TAATTAATGT AAAACTGTTT TATCTTATCA 1406

AAACAATGTA TACAACATCT CATTTAAAAA CGAGATCAAT CC                    1448
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 446 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Gly His Gln Asn Ala Ala Val Ser Glu Asn Gln Asn His Asp Asp
1               5                   10                  15

Gly Ala Ala Ser Ser Pro Gly Phe Lys Leu Val Gly Phe Ser Lys Phe
            20                  25                  30

Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val Lys Arg Phe His
        35                  40                  45

His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val Ala Arg Arg Phe
    50                  55                  60

Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser Asp Leu Ser Thr
65                  70                  75                  80

Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser Gly Asp Leu Arg
                85                  90                  95

Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser Ala Gly Glu Ile
            100                 105                 110

Lys Pro Thr Thr Thr Ala Ser Ile Pro Ser Phe Asp His Gly Ser Cys
        115                 120                 125

Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg Ala Val Ala Ile
    130                 135                 140

Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser Val Ala Asn Gly
145                 150                 155                 160

Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu Ala Val Thr Ile
                165                 170                 175

Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg Tyr Val Ser Tyr
            180                 185                 190
```

Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro Gly Phe Glu Arg
195 200 205

Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly Ile Arg Arg Leu
210 215 220

Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro Ala Leu Thr Tyr
225 230 235 240

Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu Phe Thr Ala Asp
245 250 255

Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala Val Leu Ala Ser
260 265 270

Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro Val His Gly Thr
275 280 285

Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His Asn Glu Gly Ala
290 295 300

Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile Phe Arg Thr Leu
305 310 315 320

Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe Asp Phe Met Pro
325 330 335

Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys Arg Val Gly Asp
340 345 350

Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu Leu Gly Ile Leu
355 360 365

Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile Phe Thr Lys Pro
370 375 380

Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu I[illegible] Ile Gln Arg Val Gly
385 390 3[illegible] 400

Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln Ser Gly Gly Cys
405 410 415

Gly Gly Phe Gly Lys Gly Asn Phe Ser Glu Leu Phe Lys Ser Ile Glu
420 425 430

Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val Gly
435 440 445

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 513 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Vernonia galamenensis

(vii) IMMEDIATE SOURCE:
(B) CLONE: vs1.pk0015.b2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
CCACACCGAT TGCCGGAACT TCACCGCCTC TCACGGCCTT GCAGTCCGAG CAATCGCCAT    60
TGAAGTCGAT GACGCCGAAT TAGCTTTCTC CGTCAGCGTC TCTCACGGCG CTAAACCCTC   120
CGCTGCTCCT GTAACCCTTG GAAACAACGA CGTCGTATTG TCTGAAGTTA AGCTTTACGG   180
CGATGTCGCT TTCCGGTACA TAAGTTACAA AAATCCGAAC TATACATCTT CCTTTTTGCC   240
CGGGTTCGAG CCCGTTGAAA AGACGTCGTC GTTTTATGAC CTTGACTACG GTATCCGCCG   300
TTTGGACCAC GCCGTAGGNA ACGTCCCTGA GCTTGCTTCG GCAGTGGACT ACGTGAAATC   360
ATTCACCGGA TTCCATGAGT TCGCCGAATT CACCGCGGAG GACGTCGGGA CGAGCGAGAG   420
GGAACTGAAT TCGGTCGTTT TAGCTTGCAA CAGTGAGATG GTCTTGATTC CGATGAACGA   480
GCCGGTGTAC GGAANAAAAG GAAGNAGCCA GAT                                513
```

CLAIMS

1. An isolated nucleic acid fragment encoding a plant *p*-hydroxyphenylpyruvate dioxygenase enzyme, the fragment comprising a nucleotide sequence selected from the group consisting of

nucleotide sequences encoding a polypeptide comprising the amino acid sequences set forth in SEQ ID NO:3, SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:15 and

modified nucleotide sequences essentially similar to the nucleotide sequences of SEQ ID NO:2, SEQ ID NO 10, SEQ ID NO:12 and SEQ ID NO:14 containing deletions, insertions, or substitutions in the sequence that do not affect the functional properties of the encoded protein.

2. An isolated nucleic acid fragment encoding a plant *p*-hydroxyphenylpyruvate dioxygenase enzyme, the fragment comprising a nucleotide sequence as set forth in SEQ ID NO:14.

3. A chimeric gene comprising the nucleic acid fragment of Claims 1 or 2 operably linked to at least one suitable regulatory sequence.

4. The chimeric gene of Claim 3 wherein at least one suitable regulatory sequence directs gene expression in a microorganism.

5. The chimeric gene of Claim 3 wherein the at least on itable regulatory sequence directs gene expression in a plant.

6. A plasmid vector comprising the nucleic acid fragr of Claims 1 or 2 operablylinked to at least one suitable regulatory sequence.

7. A transformed host cell comprising a host cell an plasmid vector of Claim 6.

8. The transformed host cell of Claim 7 wherein th st cell is derived from a plant or is a microorganism.

9. The transformed host cell of Claim 8 wherein the microorganism is *E. coli*.

10. A transformed plant tolerant to contact with at least one compound that inhibits the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme in a non-transformed plant, the transformed plant comprising the chimeric gene of Claim 3 and a host plant.

11. The transformed plant of Claim 10 wherein the host plant is a cereal crop plant.

12. A method to identify a compound useful for its ability to inhibit the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme comprising:

(a) transforming a host cell with the plasmid vector of Claim 6;

(b) facilitating expression of the nucleic acid fragment encoding the plant *p*-hydroxyphenylpyruvate dioxygenase enzyme:

(c) contacting the expressed enzyme from step (b) with a test compound: and

(d) evaluating the capacity of the test compound to inhibit the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme.

13. The method of Claim 12 wherein evaluating the capacity of the test compound to inhibit the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme is accomplished by measuring oxygen utilization, carbon dioxide release, homogentisate production, loss of *p*-hydroxyphenylpyruvate or maleylacetoacetate production.

14. The method of Claim 12 wherein the transformed host cell is an *E. coli* that comprises a chimeric gene encoding a plant *p*-hydroxyphenylpyruvate dioxygenase enzyme.

15. A compound that inhibits the activity of a plant *p*-hydroxyphenyl-pyruvate dioxygenase enzyme, the compound identified by the method of Claim 14.

16. A method for imparting tolerance to a plant to at least one compound that inhibits the rate of reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme comprising:

(a) transforming a host plant cell with a chimeric gene comprising a nucleic acid fragment encoding plant *p*-hydroxyphenylpyruvate dioxygenase, and

(b) expressing the chimeric gene in an amount effective to render the transformed plant substantially tolerant to the at least one compound that inhibits the rate of reaction of *p*-hydroxyphenyl-pyruvate dioxygenase.

17. A method for the microbial production of active plant *p*-hydroxy-phenylpyruvate dioxygenase enzyme comprising:

(a) stably transforming a microorganism with the chimeric gene of Claim 4 encoding the plant p-hydroxyphenylpyruvate dioxygenase;

(b) facilitating expression by the chimeric gene for a suitable period; and

(c) recovering active plant *p*-hydroxyphenylpyruvate dioxygenase enzyme.

18. A method to overexpress *p*-hydroxyphenylpyruvate dioxygenase enzyme in a plant comprising:

(a) stably transforming a host plant cell with a chimeric DNA molecule comprising at least one copy of a suitable promoter to drive expression of an associated coding sequence in a plant cell operably linked to at least one copy of a homologous or heterologous coding sequence encoding *p*-hydroxyphenyl-pyruvate dioxygenase; and

(b) growing the transformed host plant cell of step (a).

19. The method of Claim 18 wherein the chimeric DNA molecule is the chimeric gene of Claim 5.

20. An isolated nucleic acid fragment comprising a member selected from the group consisting of:

(a) an isolated nucleic acid fragment as set forth in SEQ ID NO:16;

(b) an isolated nucleic acid fragment that is essentially similar to an isolated nucleic acid fragment as set forth in SEQ ID NO:16; and

(c) an isolated nucleic acid fragment that is complementary to (a) or (b).

FIG. 1

1 CAAGAAACGNGTCGNCGACGTGCTCAGCGATGATCAGATCAAGGAGTGTGAGGAATTAGG

61 GATTCTTNTAGACAGAGATGATCAAGGGACGTTNCTTCAAATCTNCACAAAACCACTAGG

121 TGACAGGCCGACGNTATTTATAGAGATAATCCAGAGNGTAGGATGCATGATGAAAGATGT

181 GGAAGGGANGGCTTACCAGAGTGGAGNATNTNGTGGTTTTGGCAAAGGCAATT

FIG.2

1 TGAAATCA**ATG**GGCCACCAAAACGCCGCCGTTTCAGAGAATCAAAACCATGATGACGGCG
61 CTGCGTCGTCGCCGGGATTCAAGCTCGTCGGATTTTCCAAGTTCGTAAGAAAGAATCCAA
121 AGTCTGATAAATTCAAGGTTAAGCGCTTCCATCACATCGAGTTCTGGTGCGGGGACGCAA
*Eco*47III
181 CCAACGTCGCTCGTCGCTTCTCCTGGGGTCTGGGGATGAGATTCTCCGCCAAATCCGATC
241 TTTCCACCGGAAACATGGTTCACGCCTCTTACCTACTCACCTCCGGTGAACTCCGATTCC
301 TTTTCACTGCTCCTTACTCTCCGTCTCTCTCCGGCGGAGAGATTAAACCGACAACCACAG
361 GTTCTATCCCAAGTTTCGATCACGGGTCTTGTCGGTCCTTCTTCTCTTCACATGGTCTCG
421 GTGTTAGACCCGTTGCGATTGAAGTAGAAGACGCGGAGTCAGCTTTCTCCATCAGTGTAG
481 CTAATGGCGCTATTCCTTCGTCGCCTCCTATCGTCCTCAATGAAGCAGTTACGATCGCTG
541 AGGTTAAACTATACGGCGATGTTGTTCTCCGATATGTTAGTTACAAAGCAGAAGATACCG
601 AAAAATCCGAATTCTTGCCAGGGTTCGAGCGTGTAGAGGATGCGTCGTCGTTCCCATTGG
*Eco*RI
661 ATTATGGTATCCGGCGGCTTGACCACGCCGTGGGAAACGTTCCTGAGCTTGGTCCGGCTT
721 TAACTTATGTAGCGGGGTTCACTGGTTTTCACCAATTCGCAGAGTTCACAGCAGACGACG
781 TTGGAACCGCCGAGAGCGGTTTAAATTCAGCGGTCCTGGCTAGCAATGATGAAATGGTTC
*Nhe*I
841 TTCTACCGATTAACGAGCCAGTGCACGGAACAAAGAGGAAGAGTCAGATTCAGACGTATT
901 TGGAACATAACGAAGGCGCAGGGCTACAACATCTGGCTCTGATGAGTGAAGACATATTCA
961 GGACCCTGAGAGAGATGAGGAAGAGGAGCAGTATTGGAGGATTCGACTTCATGCCTTCTC
1021 CTCCGCCTACTTACTACCAGAATCTCAAGAAACGGGTCGGCGACGTGCTCAGCGATGATC
1081 AGATCAAGGAGTGTGAGGAATTAGGGATTCTTGTAGACAGAGATGATCAAGGGACGTTGC
1141 TTCAAATCTTCACAAAACCACTAGGTGACAGGCCGACGATATTTATAGAGATAATCCAGA
1201 GAGTAGGATGCATGATGAAAGATGAGGAAGGGAAGGCTTACCAGAGTGGAGGATGTGGTG
1261 GTTTTGCCAAAGGCAATTTCTCTGAGCTCTTCAAGTCCATTGAAGAATACGAAAAGACTC
1321 TTGAAGCCAAACAGTTAGTGGGA**TGA**ACAAGAAGAACCAACTAAAGGATTGTGTAAT
1381 TAATGTAAAACTGTTTTATCTTATCAAAACAATGTATACAACATCTCATTTAAAAACGAG
1441 ATCAATCC

3/6

FIG.3A

```
             1                                                  50
Arabidopsis   MGHQNAAVS ENQNHDDGAA SSFGFKLVGF SKFVRKNPKS DKFKVKRFHH
       Corn  MPPTPTAAAA GAAVAAASAA EQAAFRLVGH RNFVRFNPRS DRFHTLAFHH
        Rat                                    YWDKGPKP ERGRFLHFHS
      Mouse                                M TTYNNKGPKP ERGRFLHFHS
      Human                                M TTYSDKGAKP ERGRFLHFHS
        Pig                                M TSYSDKGEKP ERGRFLHFHS
                                                                **

             51                                                100
Arabidopsis  IEFWCGDATN VARRFSWGLG MRFSAKSDLS TGNMVHASYL LTSGDLRFLF
       Corn  VELWCADAAS AAGRFSFGLG APLAARSDLS TGNSAHASLL LRSGSLSFLF
        Rat  VTFWVGNAKQ AASFYCNKMG FEPLAYKGLE TGSREVVSHV IKQGKIVFVL
      Mouse  VTFWVGNAKQ AASFYCNKMG FEPLAYRGLE TGSREVVSHV IKRGKIVFVL
      Human  VTFWVGNAKQ AASFYCSKMG FEPLAYRGLE TGSREVVSHV IKQGKIVFVL
        Pig  VTFWVGNAKQ AASYYCSKIG FEPLAYKGLE TGSREVVSHV VKQDKIVFVF
                *   *   *       *     *   *  **     *          *

             101                                               150
Arabidopsis  TAPYSPSLSA GEIKPTTTAS IPSFDHGSCR SFFSSHGLGV RAVAIEVEDA
       Corn  TAPYAHGADA ......ATAA LPSFSAAAAR RFAADHGLAV RAVALRVADA
        Rat  CSALNPW... .......... .....NKEMG DHLVKHGDGV KDIAFEVEDC
      Mouse  CSALNPW... .......... .....NKEMG DHLVKHGDGV KDIAFEVEDC
      Human  SSALNPW... .......... .....NKEMG DHLVKHGDGV KDIAFEVEDC
        Pig  SSALNPW... .......... .....NKEMG DHLVKHGDGV KDIAFEVEDC
                                                  **  *    *  * *

             151                                               200
Arabidopsis  ESAFSISVAN GAIPSSPPIV LNEAVTIAEV KLYGDVVLRY VSYKAEDTEK
       Corn  EDAFRASVAA GARPAFGPVD LGRGFRLAEV ELYGDVVLRY VSY.PDGAAG
        Rat  EHIVQKARER GAKIVREPWV EEDKFGKVKF AVLQTYGDTT HTLVEKINYT
      Mouse  DHIVQKARER GAKIVREPWV EQDKFGKVKF AVLQTYGDTT HTLVEKINYT
      Human  DYIVQKARER GAKIMREPWV EQDKFGKVKF AVLQTYGDTT HTLVEKMNYI
        Pig  DYIVQKARER GAIIVREPWI EQDKFGKVKF AVLQTFGDTT HTLVEKMNYT
                        **     *

             201                                               250
Arabidopsis  SEFLPGFER. ..VEDASSFP LDYGIRRLDH AVGNVP..EL GPALTYVAGF
       Corn  EPFLPGFEG. ..V..ASPGA ADYGLSRFDH IVGNVP..EL APAAAYFAGF
        Rat  GRFLPGFEAP TYKDTLLPKL PSCNLEIIDH IVGNQPDQEM ESASEWYLKN
      Mouse  GRFLPGFEAP TYKDTLLPKL PRCNLEIIDH IVGNQPDQEM QSASEWYLKN
      Human  GQFLPGYEPP AFMDPLLPKL PKCSLEMIDH IVGNQPDQEM VSASEWYLKN
        Pig  GCFLPGFEAP TFTDPLLSKL PKCGLEIIDH IVGNQPDQEM ESASQWYMRN
               **** *                     **  *** *  *    *

             251                                               300
Arabidopsis  TGFHQFAEFT ADDVGTAESG LNSAVLASND EMVLLPINEP VHGTKRKSQI
       Corn  TGFHEFAEFT TEDVGTAESG LNSMVLANNS ENVLLPLNEP VHGTKRRSQI
        Rat  LQFHRFWSVD DTQVHTEYSS LRSIVVANYE ESIKMPINEP APG.RKKSQI
      Mouse  LQFHRFWSVD DTQVHTEYSS LRSIVVTNYE ESIKMPINEP APG.RKKSQI
      Human  LQFHRFWSVD DTQVHTEYSS LRSIVVANYE ESIKMPINEP APG.KKKSQI
        Pig  LQFHRFWSVD DTQIHTEYSA LRSVVMANYE ESIKMPINEP APG.KKKSQI
               ** *            *  *  * * *      *    * ***   *    ***
```

FIG.3B

```
            301                                                350
Arabidopsis QTYLEHNEGA GLQHLALMSE DIFRTLREMR KRSSIGGFDF MFSPPPTYYQ
       Corn QTFLDHHGGP GVQHMALASD DVLRTLREMQ ARSAMGGFEF MAPPTSDYYD
        Rat QEYVDYNGGA GVQHIALRTE DIITTIRHLR ER....GMEF LAVP.SSYYR
      Mouse QEYVDYNGGA GVQHIALKTE DIITAIRHLR ER....GTEF LAAP.SSYYK
      Human QEYVDYNGGA GVQHIALKTE DIITAIRHLR ER....GLEF LSVP.STYYK
        Pig QEYVDYNGGA GVQHIALKTE DIITAIRSLR ER....GVEF LAVP.FTYYK
            .       .  . .. ..    .     .     .    .  .    .   ..

            351                                                400
Arabidopsis NLKK..RVGD VLSDDQIKEC EELGILVDRD DQGTLLQIFT KPLGDRPTIF
       Corn GVRR..RAGD VLTEAQIKEC QELGVLVDRD DQGVLLQIFT KPVGDRPTLF
        Rat LLRENLKTSK IQVKENMDVL EELKILVDYD EKGYLLQIFT KPMQDRPTLF
      Mouse LLRENLKSAK IQVKESMDVL EELHILVDYD EKGYLLQIFT KPMQDRPTLF
      Human QLREKLKTAK IKVKENIDAL EELKILVDYD EKGYLLQIFT KPVQDRPTLF
        Pig QLQEKLKSAK IRVKESIDVL EELKILVDYD EKGYLLQIFT KPMQDRPTVF
                                   **  *** *   * ****** **  **** *

            401                                                450
Arabidopsis IEIIQRVGCM MKDEEGKAYQ SGGCGGFGKG NFSELFKSIE EYEKTLEAKQ
       Corn LEIIQRIGCM EKDEKGQEYQ KGGCGGFGKG NFSQLFKSIE DYEKSLEAKQ
        Rat LEVIQRHNHQ .......... .....GFGAG NFNSLFKAFE E.EQALRG
      Mouse LEVIQRHNHQ .......... .....GFGAG NFNSLFKAFE E.EQALRGNL
      Human LEVIQRHNHQ .......... .....GFGAG NFNSLFKAFE E.EQNLRGNL
        Pig LEVIQRNNHQ .......... .....GFGAG NFNSLFKAFE E.EQELRGNL
             * ***                     ***   **  ***  *   *  *

            451        462
Arabidopsis LVG                   (Seq. I.D. No. 15)
       Corn AAAAAAAQGS            (Seq. I.D. No. 11)
        Rat                       (Seq. I.D. No.  9)
      Mouse TDLEPNGVRS GM         (Seq. I.D. No.  8)
      Human TNMETNGVVP GM         (Seq. I.D. No.  6)
        Pig TDTDPNGVPF RL         (Seq. I.D. No.  7)
```

FIG.4

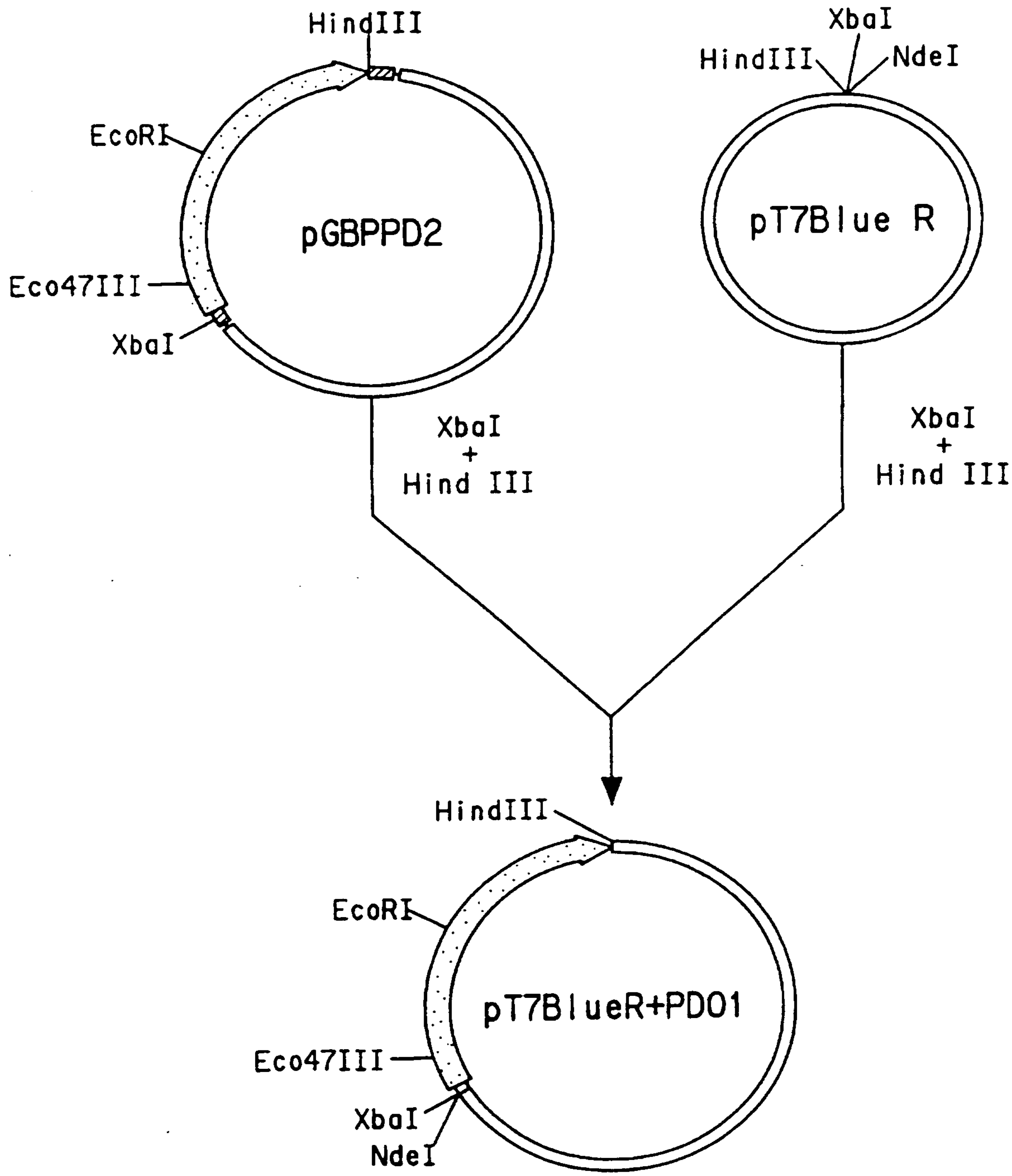
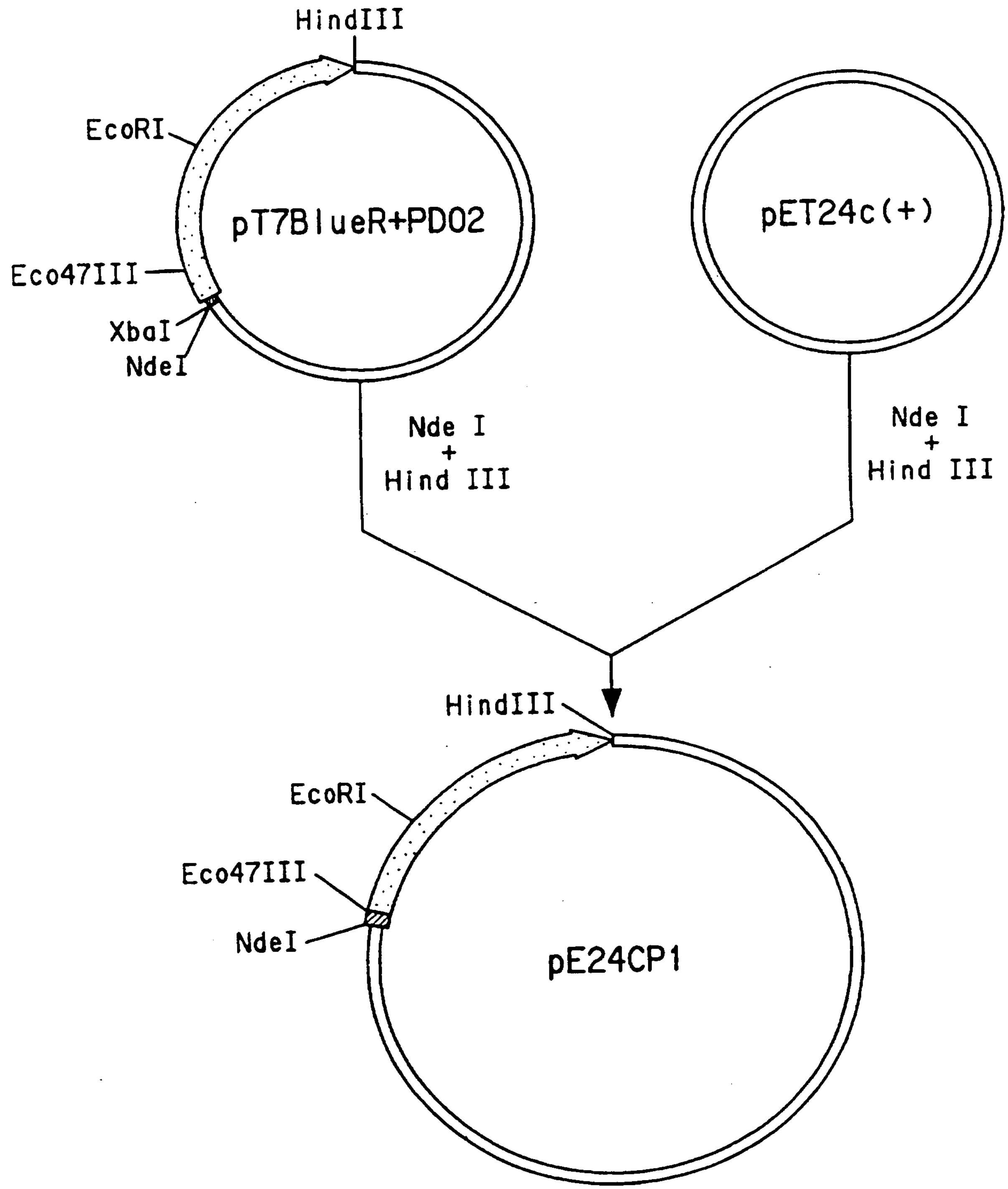

HindIII
EcoRI
pGBPPD2
Eco47III
XbaI
XbaI
HindIII
NdeI
pT7Blue R
XbaI
+
Hind III
XbaI
+
Hind III
HindIII
EcoRI
pT7BlueR+PD01
Eco47III
XbaI
NdeI

FIG.5


HindIII
EcoRI
pT7BlueR+PD02
Eco47III
XbaI
NdeI
pET24c(+)
Nde I
+
Hind III
Nde I
+
Hind III
HindIII
EcoRI
Eco47III
NdeI
pE24CP1

INTERNATIONAL SEARCH REPORT

Intern al Application No
PCT/US 97/11295

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/82 C12Q1/26 C12Q1/02 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEWMAN, T., ET AL.: "2960 Arabidopsis thaliana cDNA clone 91B13T7" EMBL SEQUENCE DATABASE, REL. 40, 16-JUN-1994, ACCESSION NO. T20952, XP002028637 see sequence ---	1,2
X	NEWMAN, T., ET AL.: "20804 Arabidopsis thaliana cDNA clone 231K20T7" EMBL SEQUENCE DATABASE, REL.47, 8-MAR-1996, ACCESSION NO. N65764, XP002029449 see sequence ---	1,2
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

4

Date of the actual completion of the international search	Date of mailing of the international search report
26 September 1997	07.10.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHULZ A ET AL: "SC-0051, A 2-BENZOYL-CYCLOHEXANE-1,3-DIONE BLEACHING HERBICIDE, IS APOTENT INHIBITOR OF THE ENZYME P-HYDROXYPHENYLPYRUVATE DIOXYGENASE" FEBS LETTERS, vol. 318, no. 2, March 1993, pages 162-166, XP002028049 see the whole document ---	15
X	BARTA I C ET AL: "BENZOYLCYCLOHEXANEDIONE HERBICIDES ARE STRONG INHIBITORS OF PURIFIED P-HYDROXYPHENYLPYRUVIC ACID DIOXYGENASE OF MAIZE" PESTICIDE SCIENCE, vol. 45, no. 3, 1 November 1995, page 286/287 XP000547268 see the whole document ---	15
X	EP 0 614 970 A (HOECHST SCHERING AGREVO GMBH) 14 September 1994 see the whole document ---	15
P,X	WO 96 38567 A (RHONE POULENC AGROCHIMIE ;SAILLAND ALAIN (FR); ROLLAND ANNE (FR);) 5 December 1996 see sequence ID no. 2 ---	1,2
P,X	BARTLEY, G.E., ET AL.: "Arabidopsis thaliana p-hydroxyphenylpyruvate dioxygenase (HPD) mRNA, complete cds." EMBL SEQUENCE DATABASE, REL. 51, 19-MAR-1997, ACCESSION NO. U89267, XP002041908 see sequence ---	1,2,20
A	EP 0 652 286 A (RHONE POULENC AGROCHIMIE) 10 May 1995 see page 7, line 35 - line 47 ---	10,16,18
A	MISAWA N ET AL: "EXPRESSION OF AN ERWINA PHYTOENE DESATURASE GENE NOT ONLY CONFERS MULTIPLE RESISTANCE TO HERBICIDES INTERFERING WITH CAROTENOID BIOSYNTHESIS BUT ALSO ALTERS XANTHOPHYLL METABOLISM IN TRANSGENIC PLANTS" PLANT JOURNAL, vol. 6, no. 4, 1994, pages 481-489, XP002017203 see the whole document ---	10,16,18

-/--

4

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DENOYA C D ET AL: "A STREPTOMYCES AVERMITILIS GENE ENCODING A 4-HYDROXYPHENYLPYRUVIC ACID DIOXYGENASE-LIKE PROTEIN THAT DIRECTS THE PRODUCTION OF HOMOGENTISIC ACID AND AN OCHRONOTIC PIGMENT IN ESCHERICHIA COLI" JOURNAL OF BACTERIOLOGY, vol. 176, no. 17, September 1994, pages 5312-5319, XP002028042 see the whole document ---	17
A	NORRIS, S.R., ET AL.: "Gnetic dissection of carotenoid synthesis in Arabidopsis defines plastoquinone as an essential component of phytoene desaturation" THE PLANT CELL, vol. 7, December 1995, pages 2139-2149, XP002041909 cited in the application see the whole document -----	1-20

4

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/11295

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0614970 A	14-09-94	DE 4305696 A	01-09-94
		CA 2116421 A	26-08-94
		JP 6343464 A	20-12-94
WO 9638567 A	05-12-96	FR 2734840 A	06-12-96
		FR 2734841 A	06-12-96
		FR 2734842 A	06-12-96
		AU 6228696 A	18-12-96
EP 0652286 A	10-05-95	FR 2712302 A	19-05-95
		AU 7775194 A	18-05-95
		BG 99169 A	28-07-95
		BR 9404562 A	20-06-95
		CA 2135461 A	11-05-95
		CN 1121958 A	08-05-96
		CZ 9402743 A	13-09-95
		HU 70464 A	30-10-95
		JP 7184664 A	25-07-95
		NZ 264879 A	28-10-96
		PL 305775 A	15-05-95
		SK 134094 A	07-06-95
		US 5635618 A	03-06-97
		ZA 9408826 A	17-07-95

CORRECTED VERSION*

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/53, 15/82, C12Q 1/26, 1/02, A01H 5/00 — A1

(11) International Publication Number: WO 97/49816

(43) International Publication Date: 31 December 1997 (31.12.97)

(21) International Application Number: PCT/US97/11295

(22) International Filing Date: 26 June 1997 (26.06.97)

(30) Priority Data: 60/021,364 27 June 1996 (27.06.96) US

(71) Applicant (*for all designated States except US*): E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): MAXWELL, Carl, Arthur [US/US]; 35 Mary Anita Court, Elkton, MD 21921 (US). SCOLNIK, Pablo, Ariel [US/US]; 120 Spottswood Lane, Kennett Square, PA 19348 (US). WITTENBACH, Vernon, Arie [US/US]; 609 Greenbank Road, Wilmington, DE 19808 (US). GUTTERIDGE, Steven [GB/US]; 4 Austin Road, Wilmington, DE 19810 (US).

(74) Agent: FLOYD, Linda, A.; E.I. du Pont de Nemours and Company, Legal Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KG, KP, KR, KZ, LC, LK, LR, LT, LV, MD, MG, MK, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, SL, TJ, TM, TR, TT, UA, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: PLANT GENE FOR *P*-HYDROXYPHENYLPYRUVATE DIOXYGENASE

```
1      CAAGAAACGNGTCGNCGACGTGCTCAGCGATGATCAGATCAAGGAGTGTGAGGAATTAGG
61     GATTCTTNTAGACAGAGATGATCAAGGGACGTTNCTTCAAATCTNCACAAAACCACTAGG
121    TGACAGGCCGACGNTATTTATAGAGATAATCCAGAGNGTAGGATGCATGATGAAAGATGT
181    GGAAGGGANGGCTTACCAGAGTGGAGNATNTNGTGGTTTTGGCAAAGGCAATT
```


(57) **Abstract**

The invention relates to the isolation and modification of nucleic acid sequences encoding *p*-hydroxyphenylpyruvate dioxygenase enzyme from plants. These nucleic acid sequences were used to establish methods of identification of new herbicidal compounds that inhibit the activity of this enzyme, and to prepare new crop plants that are tolerant to the herbicidal action of inhibitors of this enzyme. Chimeric genes comprising nucleic acid fragments containing all or part of the nucleic acid sequences encoding *p*-hydroxyphenylpyruvate dioxygenase may be used to produce active plant *p*-hydroxyphenylpyruvate dioxygenase enzyme in microorganisms, and to cause the production of modified forms of the enzyme in plants that may render such plants tolerant to inhibitors of the enzyme.

*(Referred to in PCT Gazette No. 12/1998, Section II)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania
AM	Armenia
AT	Austria
AU	Australia
AZ	Azerbaijan
BA	Bosnia and Herzegovina
BB	Barbados
BE	Belgium
BF	Burkina Faso
BG	Bulgaria
BJ	Benin
BR	Brazil
BY	Belarus
CA	Canada
CF	Central African Republic
CG	Congo
CH	Switzerland
CI	Côte d'Ivoire
CM	Cameroon
CN	China
CU	Cuba
CZ	Czech Republic
DE	Germany
DK	Denmark
EE	Estonia
ES	Spain
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
GE	Georgia
GH	Ghana
GN	Guinea
GR	Greece
HU	Hungary
IE	Ireland
IL	Israel
IS	Iceland
IT	Italy
JP	Japan
KE	Kenya
KG	Kyrgyzstan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
KZ	Kazakstan
LC	Saint Lucia
LI	Liechtenstein
LK	Sri Lanka
LR	Liberia
LS	Lesotho
LT	Lithuania
LU	Luxembourg
LV	Latvia
MC	Monaco
MD	Republic of Moldova
MG	Madagascar
MK	The former Yugoslav Republic of Macedonia
ML	Mali
MN	Mongolia
MR	Mauritania
MW	Malawi
MX	Mexico
NE	Niger
NL	Netherlands
NO	Norway
NZ	New Zealand
PL	Poland
PT	Portugal
RO	Romania
RU	Russian Federation
SD	Sudan
SE	Sweden
SG	Singapore
SI	Slovenia
SK	Slovakia
SN	Senegal
SZ	Swaziland
TD	Chad
TG	Togo
TJ	Tajikistan
TM	Turkmenistan
TR	Turkey
TT	Trinidad and Tobago
UA	Ukraine
UG	Uganda
US	United States of America
UZ	Uzbekistan
VN	Viet Nam
YU	Yugoslavia
ZW	Zimbabwe

TITLE

PLANT GENE FOR *P*-HYDROXYPHENYLPYRUVATE DIOXYGENASE

FIELD OF THE INVENTION

This invention relates to the isolation and modification of nucleic acid encoding *p*-hydroxyphenylpyruvate dioxygenase enzyme from plants. These nucleic acid sequences were used to establish methods of identification of new herbicidal compounds that inhibit the activity of this enzyme, and to prepare new crop plants that are tolerant to the herbicidal action of inhibitors this enzyme. Chimeric genes comprising nucleic acid fragments containing all or part of the nucleic acid sequences encoding *p*-hydroxyphenylpyruvate dioxygenase may be used to produce active plant *p*-hydroxyphenylpyruvate dioxygenase enzyme in microorganisms, and to cause the production of modified forms of the enzyme in plants that may render such plants tolerant to inhibitors of the enzyme.

BACKGROUND OF THE INVENTION

Bleaching herbicides affect plant chloroplasts by decreasing their chlorophyll and carotenoid content. Several bleaching herbicides are known to inhibit the enzyme phytoene desaturase, resulting in the accumulation of phytoene in treated plants. However, compounds of the benzoyl cyclohexane-1,3-dione type cause the accumulation of phytoene in plants but are not inhibitors of phytoene desaturase *in vitro* (Sandmann, G., et al. (1990) *Pestic. Sci.* 30:353-355). Subsequent work revealed that these compounds are effective inhibitors of *p*-hydroxyphenylpyruvate dioxygenase (*p*-hydroxyphenylpyruvate:oxygen oxidoreductase EC 1.13.11.27), a key enzyme in the biosynthesis of plastoquinones and tocopherols (Schulz, A., et al. (1993) *FEBS Lett.* 318:162-166). Based on the observation that phytoene desaturase requires a quinone as an electron acceptor, these authors postulated that by inhibiting *p*-hydroxyphenylpyruvate dioxygenase, these herbicides act indirectly on phytoene desaturase by blocking the biosynthesis of quinones.

The proposal that *p*-hydroxyphenylpyruvate dioxygenase is essential for carotenoid biosynthesis has received support from genetic studies in the plant model system *Arabidopsis thaliana*. Mutations in the *pds1* and *pds2* genetic loci result in mutant plants that accumulate phytoene. However, genetic mapping of these mutant genes indicates that they do not correspond to the gene encoding the enzyme phytoene desaturase. The *pds1* mutation can be rescued by homogentisic acid, the substrate of *p*-hydroxyphenylpyruvate dioxygenase. Therefore, this mutation corresponds to a defect in the activity of *p*-hydroxyphenylpyruvate dioxygenase (Norris, S. R., et al. (1995) *Plant Cell* 7:2139-2149).

In light of these disclosures. *p*-hydroxyphenylpyruvate dioxygenase is a promising new target for new herbicidal compounds. Research aimed at discovering new herbicides based on this mode of action would be greatly facilitated by the isolation of the plant gene encoding this enzyme and by the functional expression of this gene in transgenic organisms. For example. active enzyme produced in recombinant microorganisms could be used to establish screening methods for the identification of novel active compounds and to obtain structural and mechanistic information useful to guide further chemical synthesis. Furthermore, isolation of this gene would facilitate research aimed at generating mutant. herbicide-tolerant versions of the enzyme that may confer herbicide resistance to transgenic plants.

A partial sequence of an *Arabidopsis thaliana* cDNA with homology to corresponding mammalian sequences encoding *p*-hydroxyphenylpyruvate dioxygenase has been identified (GenBank Accession No. T20952). but this truncated sequence is insufficient to identify an active plant *p*-hydroxyphenylpyruvate dioxygenase. WO 96/38567 A2 addresses the utility that would be attached to a DNA sequence of a *p*-hydroxyphenylpyruvate dioxygenase gene. but there is no biochemical evidence of function associated with the sequences disclosed.

SUMMARY OF THE INVENTION

This invention pertains to the isolation and characterization of nucleic acid fragments encoding plant *p*-hydroxyphenylpyruvate dioxygenase enzymes. More specifically, this invention pertains to isolated nucleic acid fragments encoding the *p*-hydroxyphenylpyruvate dioxygenase enzymes from *Arabidopsis thaliana* and *Zea mays*.

This invention also pertains to the production of active plant *p*-hydroxyphenylpyruvate dioxygenase enzyme in *E. coli*. In one embodiment, a chimeric gene comprising a nucleic acid fragment encoding a polypeptide that possesses *p*-hydroxyphenylpyruvate dioxygenase activity, operably linked to regulatory sequences that direct gene expression in *E. coli*, is claimed. In another embodiment, a plasmid vector comprising said chimeric gene is disclosed. In yet another embodiment, a transformed *E. coli* comprising a chimeric gene consisting of a nucleic acid fragment encoding a polypeptide that possesses *p*-hydroxyphenylpyruvate dioxygenase activity is disclosed.

This invention also pertains to a method of identifying substances that inhibit the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme. In one embodiment, the invention pertains to an assay for the detection of inhibitors of *p*-hydroxyphenylpyruvate dioxygenase wherein a polypeptide

derived from a transformed *E. coli* that displays *p*-hydroxyphenylpyruvate dioxygenase activity is incubated in the presence of a test substance. Following incubation, *p*-hydroxyphenylpyruvate dioxygenase enzymatic activity is measured wherein a reduction of enzymatic activity is indicative of the inhibitory capacity of the test substance. Enzymatic activity can be measured by any appropriate means, including but not limited to oxygen utilization, carbon dioxide release, homogentisate production, and loss of *p*-hydroxyphenylpyruvate. Results are quantified by radiometric, colorimetric or chromatographic means.

In another embodiment, this invention pertains to plants that are substantially tolerant to the application of at least one compound that inhibits the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase. Plants may be rendered tolerant by overexpression of the wild-type *p*-hydroxyphenylpyruvate dioxygenase, by expression of a naturally-occuring resistant variant of this enzyme, or by expression of an altered form of *p*-hydroxyphenylpyruvate dioxygenase that is resistant to the action of compounds that are inhibitory to the wild-type enzyme.

A further embodiment of the invention is an isolated nucleic acid fragment comprising a member selected from the group consisting of:

(a) an isolated nucleic acid fragment as set forth in SEQ ID NO:16;

(b) an isolated nucleic acid fragment that is essentially similar to an isolated nucleic acid fragment as set forth in SEQ ID NO:16;. and

(c) an isolated nucleic acid fragment that is complementary to (a) or (b).

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE DESCRIPTIONS

The invention can be more fully understood from the following detailed description and the accompanying drawings and the sequence descriptions which form a part of this application.

Figure 1 presents a partial nucleic acid sequence of an expressed sequence tag (EST) bearing GenBank Accession No. T92052 obtained from an *Arabidopsis thaliana* cDNA library. This sequence was contained in clone 91B13T7 of the library.

Figure 2 presents the nucleic acid sequence of the cloned cDNA encoding a full-length form of *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme, as it was initially determined (SEQ ID NO:2). Translation start and stop codons are underlined. Selected restriction sites are indicated.

Figure 3 presents the amino acid sequence comparison between full-length *p*-hydroxyphenylpyruvate dioxygenases from *Arabidopsis thaliana* (SEQ ID NO:15) and *Zea mays* (SEQ ID NO:11) and the *p*-hydroxyphenylpyruvate dioxygenase enzymes derived from human (SEQ ID NO:6, GenBank Acc. No. U29895), pig (SEQ ID NO:7, GenBank Acc. No. D13390), mouse (SEQ ID NO:8, GenBank Acc. No. D29987) and rat (SEQ ID NO:9, GenBank Acc. No. M18405). Asterisks indicate amino acid residues that are conserved across all six species. This figure was created using the Pileup program of GCG (Program Manual for the Wisconsin Package, Version 9.0-OpenVMS, December 1996, Genetics Computer Group, 575 Science Drive, Madison, WI, USA 53711).

Figure 4 is a diagram describing the construction of the intermediate plasmid vector pT7BlueR + PDO1.

Figure 5 is a diagram describing the construction of *E. coli* expression vector pE24CP1.

Applicants have provided a sequence listing in conformity with "Rules for the Standard Representation of Nucleotide and Amino Acid Sequences in Patent Applications" (Annexes I and II to the Decision of the President of the EPO, published in Supplement No. 2 to OJ EPO, 12/1992) and with 37 C.F.R. 1.821-1.825 and Appendices A and B ("Requirements for Application Disclosures Containing Nucleotides and/or Amino Acid Sequences").

SEQ ID NO:1 presents a partial nucleic acid sequence of an expressed sequence tag (EST) bearing GenBank Accession No. T92052 obtained from an *Arabidopsis thaliana* cDNA library. This sequence was contained in clone 91B13T7 of the library.

SEQ ID NO:2 presents the initial determination of the nucleic acid sequence and the deduced amino acid sequence of a cDNA encoding a full-length form of *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme, as contained in plasmid pGBPPD2.

SEQ ID NO:3 presents the initially deduced amino acid sequence encoded by a cDNA for *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme.

SEQ ID NOS:4 and 5 present the nucleotide sequences of a pair of complementary oligonucleotides (CAM 32 and CAM 33, respectively) used to facilitate subcloning and expression of the gene encoding *p*-hydroxyphenylpyruvate dioxygenase without the chloroplast transit sequence.

SEQ ID NO:6 presents the amino acid sequence of *p*-hydroxyphenylpyruvate dioxygenase enzyme derived from human (GenBank Acc. No. U29895).

SEQ ID NO:7 presents the amino acid sequence of *p*-hydroxyphenylpyruvate dioxygenase enzyme derived from pig (GenBank Acc. No. D13390).

SEQ ID NO:8 presents the amino acid sequence of *p*-hydroxyphenylpyruvate dioxygenase enzyme derived from mouse (GenBank Acc. No. D29987).

SEQ ID NO:9 presents the amino acid sequence of *p*-hydroxyphenylpyruvate dioxygenase enzyme derived from rat (GenBank Acc. No. M18405).

SEQ ID NO:10 presents the nucleic acid sequence and deduced amino acid sequence of the cloned cDNA encoding the *Zea mays p*-hydroxyphenylpyruvate dioxygenase enzyme, as contained in plasmid pMPDO.

SEQ ID NO:11 presents the deduced amino acid sequence of the cloned cDNA encoding the *Zea mays p*-hydroxyphenylpyruvate dioxygenase enzyme, as contained in plasmid pMPDO.

SEQ ID NO:12 presents the nucleic acid sequence and the deduced amino acid sequence of the truncated form of *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme as contained in pE24CP1.

SEQ ID NO:13 presents the deduced amino acid sequence of the truncated form of *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme as contained in pE24CP1.

SEQ ID NO:14 presents the revised nucleic acid sequence and the deduced amino acid sequence of the cloned cDNA encoding the full-length *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme, as contained in plasmid pGBPPD2.

SEQ ID NO:15 presents the revised amino acid sequence deduced from the cDNA for the full length *Arabidopsis thaliana p*-hydroxyphenylpyruvate dioxygenase enzyme.

SEQ ID NO:16 presents the nucleic acid sequence determined from a portion of a cDNA from *Vernonia galamenensis*, as contained in clone vs1.pk0015.b2.

DETAILS OF THE INVENTION

BIOLOGICAL DEPOSITS

The following biological materials have been deposited under the terms of the Budapest Treaty at American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, MD 20852, and bear the following accession numbers:

Depositor Identification		Int'l. Depository	
Host Strain	Plasmid	Accession Number	Date of Deposit
E. coli BL21(DE3)	pE24CP1	ATCC 98083	June 25, 1996
N/A	pGBPPD2	ATCC 97622	June 25, 1996
N/A	pMPDO	ATCC 209120	June 12, 1997

Definitions

In the context of this disclosure, a number of terms shall be utilized. As used herein, the term "nucleic acid" refers to a large molecule which can be single-stranded or double-stranded, composed of monomers (nucleotides) containing a sugar, phosphate and either a purine or pyrimidine. A "nucleic acid fragment" is a portion of a given nucleic acid molecule. As used herein, "DNA" (deoxyribonucleic acid) is the genetic material, whereas "RNA" (ribonucleic acid) is involved in the transfer of the information encoded by the DNA into proteins and polypeptides. A "genome" is the entire body of genetic material contained in each cell of an organism. The term "nucleotide sequence" refers to a polymer of DNA or RNA which can be single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases capable of incorporation into DNA or RNA polymers.

As used herein, "essentially similar" refers to DNA sequences that may involve base changes that do not cause a change in the encoded amino acid or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. It is therefore understood that the invention encompasses more than the specific exemplary sequences. Modifications to the sequence, such as deletions, insertions, or substitutions in the sequence which produce "silent changes" (i.e., those that do not substantially affect the functional properties of the resulting protein molecule) are also contemplated. For example, alteration(s) in the gene sequence which reflects the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated; thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be

expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence in order to study the effect of alteration on the biological activity of the protein. Each of the proposed modifications is well within the routine skill in the art, as is determination of retention of biological activity of the encoded products. Moreover, the skilled artisan recognizes that "essentially similar" sequences encompassed by this invention are also defined by their ability to hybridize, under stringent conditions (0.1X SSC, 0.1% SDS, 65°C), with the sequences exemplified herein.

"Gene" refers to a nucleic acid fragment that encodes a specific protein, including regulatory sequences preceding (5' non-coding) and following (3' non-coding) the coding region. "Native" gene refers to the gene as found in nature with its own regulatory sequences. "Chimeric" gene refers to a gene comprising heterogeneous regulatory and coding sequences. "Endogenous" gene refers to the native gene normally found in its natural location in the genome. A "foreign" gene refers to a gene not normally found in the host organism but that is introduced by gene transfer.

"Coding sequence" refers to a DNA sequence that codes for a specific protein and excludes the non-coding sequences.

"Initiation codon" and "termination codon" refer to a unit of three adjacent nucleotides in a coding sequence that specifies initiation and termination, respectively, of protein synthesis (mRNA translation). "Open reading frame" refers to the amino acid sequence encoded between translation initiation and termination codons of a coding sequence.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript or it may be a RNA sequence derived from posttranscriptional processing of the primary transcript. "Messenger RNA" (mRNA) refers to RNA that can be translated into protein by the cell. "cDNA" refers to a double-stranded DNA, one strand of which is complementary to and derived from mRNA by reverse transcription. "Sense RNA" refers to RNA transcript that includes the mRNA.

As used herein, "regulatory sequences" are nucleotide sequences that control the transcription or expression of a coding sequence located upstream (5'), within, or downstream (3') to the coding sequence, act in conjunction with the protein biosynthetic apparatus of the cell and include promoters, translation leader sequences, transcription termination sequences, and polyadenylation sequences.

"Promoter" refers to a DNA sequence in a gene, usually upstream (5') to its coding sequence, which controls the expression of the coding sequence by providing the recognition for RNA polymerase and other factors required for proper transcription. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions. In the case of eukaryotic organisms, it may also contain enhancer elements.

An "enhancer element" is a DNA sequence which can stimulate promoter activity. It may be an innate element of the promoter or a heterologous element inserted to enhance the activity level and tissue-specificity of a promoter. "Constitutive promoters" refer to those enhancer elements that direct gene expression in all tissues and at all times. "Organ-specific" or "development-specific" promoters as referred to herein are those that direct gene expression almost exclusively in specific organs, such as leaves or seeds, or at specific development stages in an organ, such as in early or late embryogenesis, respectively.

The term "operably linked" refers to nucleic acid sequences on a single nucleic acid molecule which are associated so that the function of one is affected by the other. For example, a promoter is operably linked with a structural gene (i.e., a gene encoding *p*-hydroxyphenylpyruvate dioxygenase, as disclosed herein) when it is capable of affecting the expression of that structural gene (i.e., that the structural gene is under the transcriptional control of the promoter).

The term "expression", as used herein, is intended to mean the production of the protein product encoded by a gene. More particularly, "expression" refers to the transcription and stable accumulation of the sense RNA (mRNA) derived from the nucleic acid fragment(s) of the invention that, in conjuction with the protein apparatus of the cell, results in altered levels of protein product. "Overexpression" refers to the production of a gene product in transgenic organisms that exceeds levels of production in normal or non-transformed organisms. "Altered levels" refers to the production of gene product(s) in transgenic organisms in amounts or proportions that differ from that of normal or non-transformed organisms. "Facilitating expression" refers to steps and conditions for culturing host cells containing the desirable gene to yield an increased production of the enzyme. For example, addition of a chemical inducer specific to the particular promoter operably linked to the gene facilitates expression of the encoded enzyme. This is measured relative to the production levels of an untreated gene.

The "3' non-coding sequences" refers to the DNA sequence portion of a gene that contains a polyadenylation signal and any other regulatory signal capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor.

The "translation leader sequence" refers to that DNA sequence portion of a gene between the promoter and coding sequence that is transcribed into RNA and is present in the fully processed mRNA upstream (5') of the translation start codon. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability, or translation efficiency.

"Transformation" herein refers to the transfer of a foreign gene into the genome of a host organism and its genetically stable inheritance. Bacterial transformation can proceed by any of several methods well known in the art, including calcium chloride-mediated transformation and electroporation. Examples of methods of plant transformation include *Agrobacterium*-mediated transformation and particle-accelerated or "gene gun" transformation technology (U.S. Patent No. 4,945,050).

"Host cell" refers to the cell that is transformed with the introduced genetic material.

"Plasmid vector" refers to a double-stranded, closed circular, extra-chromosomal DNA molecule.

"Tolerant" or "tolerance" refers to a condition whereby a cell or an organism is able to withstand the effect of application of a compound or composition at a concentration or application rate that causes a demonstrable effect in or against cells or organisms that are not tolerant. For example, the growth or survival of a plant that is tolerant to application of a herbicidal compound or composition will be less affected than the growth or survival of a plant that is not tolerant to application of the herbicidal compound or composition.

Cloning of Plant Genes Encoding *p*-Hydroxyphenylpyruvate Dioxygenase

The *p*-hydroxyphenylpyruvate dioxygenases from plants are a promising new class of targets for new herbicidal compounds. In order to be able to study this enzyme in detail, and to have available supplies of enzyme for inhibitor screening, cDNA clones encoding plant *p*-hydroxyphenylpyruvate dioxygenases were identified. These nucleic acid fragments are useful for the production of their encoded enzymes, for isolation of clones from additional plant sources that encode other *p*-hydroxyphenylpyruvate dioxygenase enzymes, and for understanding the biochemical and structural properties of these enzymes.

Nucleic acid fragments comprising nucleotide sequences that encode different forms of the enzyme *p*-hydroxyphenylpyruvate dioxygenase from the plant *Arabidopsis thaliana* have now been isolated. Subsequently, these nucleotide sequences were expressed in *E. coli* cells and shown to direct the synthesis of plant *p*-hydroxyphenylpyruvate dioxygenase enzymes.

An automated search of nucleotide sequences contained in a database representing an *Arabidopsis* cDNA library for sequences homologous to other known, non-plant *p*-hydroxyphenylpyruvate dioxygenase genes revealed the plasmid cDNA clone 91B13T7. This cDNA was obtained from the Arabidopsis Seed Stock Center at Ohio State University. Plasmid DNA suitable for nucleotide sequence determination was prepared and the nucleotide sequence of the plasmid insert was determined. The resulting sequence was not interpretable, suggesting possible contamination of the plasmid sample by an extraneous nucleic acid. This assumption was confirmed by digesting the plasmid DNA sample with restriction enzymes and separating the resulting nucleic acid fragments by agarose gel electrophoresis. This analysis revealed the presence of nucleic acid fragments that could not be derived from the plasmid carrying the putative *p*-hydroxyphenyl-pyruvate dioxygenase fragment. Furthermore, a search of the publically available nucleic acid sequence databases revealed that the *Arabidopsis thaliana* sequence reported for cDNA clone 91B13T7 corresponded to a truncated cDNA (Figure 1). Based on publically available mammalian cDNA sequence information for *p*-hydroxyphenylpyruvate dioxygenase, the minimum length expected for a cDNA encoding a complete *p*-hydroxyphenylpyruvate dioxygenase enzyme is 1 kb (Table 1).

Table 1

Predicted cDNA Length for Sequences Encoding *p*-Hydroxyphenylpyruvate Dioxygenase

Organism	Amino Acid Residues	Minimum cDNA (kb)
Human	392	1.176
Pig	392	1.176
Pseudomonas sp.	357	1.071

Therefore, based on the expected length of a cDNA capable of encoding a functional *p*-hydroxyphenylpyruvate dioxygenase, the *Arabidopsis thaliana* sequence obtained from the public database was insufficient to encode a full-length, active *p*-hydroxyphenylpyruvate dioxygenase enzyme. Therefore, a cDNA with the capacity to encode a full-length enzyme *Arabidopsis thaliana* was cloned.

as described herein. A 400 bp segment of the insert of plasmid 91B13T7 was liberated by digestion with restriction enzymes and used to screen a cDNA library prepared from norflurazon-treated *Arabidopsis thaliana* seedlings (Scolnik, P. A., and Bartley, G. E. (1994) *Plant Physiol.* 104:1469-1470). Several clones showing positive hybridization to this probe were sequenced. The initial determination of the sequence of the longest cDNA clone obtained from this effort is shown in Figure 2 and in SEQ ID NO:2. During the course of subsequent work with this clone,it became necessary to confirm certain features of the sequence. A corrected sequence of this cDNA is presented in SEQ ID NO:12.

The sequence reported in Figure 2 indicates that this cDNA has the capacity to encode a protein of MW 48,841 which, as shown in Figure 3, has a high level of homology to *p*-hydroxyphenylpyruvate dioxygenase enzymes from other eukaryotes.

. A cDNA capable of encoding a full-length *p*-hydroxyphenylpyruvate dioxygenase has also been obtained from corn. This cDNA, contained in plasmid pMPDO, was identified in a corn cDNA library using an approximately 900 base pairs portion of the *Arabidopsis* cDNA as a probe. The predicted amino acid sequence that is encoded by the corn cDNA is also compared to *p*-hydroxyphenylpyruvate dioxygenase enzymes from other eukaryotes in Figure 3.

A cDNA library was prepared from messenger RNA isolated from developing seeds of *Vernonia galamenensis*. Random sequencing of the clones contained in the library identified a probable clone, designated vs1.pk0015.b2, for the *p*-hydroxyphenylpyruvate dioxygenase from this plant. The 513 bp expressed sequence tag (EST) is presented in SEQ ID NO:16.

<u>Expression of the *Arabidopsis thaliana* cDNA Encoding *p*-Hydroxyphenylpyruvate Dioxygenase in *E. coli*</u>

The nucleic acid fragments of the instant invention encoding a plant *p*-hydroxyphenylpyruvate dioxygenase enzymes can be operably linked to suitable regulatory sequences, thereby creating chimeric genes that can be used to direct expression of the enzyme in transgenic organisms. These transgenic organisms include, but are not limited to: plants (*Plant Molecular Biology*; Croy, R. R. D., Ed.; Bios Scientific Publishers; 1993); microorganisms, including *Escherichia coli* (Gold, L. (1990) *Methods in Enzymology* 185:11), *Bacillus subtilis* (Henner, D. J. (1990) *Methods in Enzymology* 185:199), yeast (Gellissen, G., et al. (1992) *Antonie Leeuwenhoek* 62:79), and fungi, including members of the genus *Aspergillus* (Devchand, M. and Gwynne, D. I. (1991) *J. Biotechnol.* 17:3); and insect cells containing recombinant baculoviruses (Lukow, V. A. and Summers, M. D. (1988) *Bio/Technology* 6:47).

One skilled in the art can isolate the coding sequences from the fragments of the invention by using or creating sites for restriction endonucleases, as described in Sambrook, J., et al.((1989) *Molecular Cloning, A Laboratory Manual*, 2nd ed.; Cold Spring Harbor Laboratory Press; hereinafter "Maniatis"). Alternatively, polymerase chain reaction (PCR) techniques can be employed to isolate and/or modify the fragments of the invention (Newton, C. R. and Graham, A. (1994) *PCR*; Bios Scientific Publishers).

Arabidopsis p-hydroxyphenylpyruvate dioxygenase was expressed in *E. coli* under control of a T7 promoter in a strain expressing T7 RNA polymerase (Studier, F. W., et al. (1990) *Methods in Enzymology* 185:60). Promoters other than T7 are commonly used in expression vectors and could be substituted for protein expression in *E. coli*. Examples of alternative promoters include, but are not limited to, *trp* (Yansura, D. G. and Henner, D. J. (1990) *Methods in Enzymology* 185:54), P_L (Remaut, E. et al. (1981) *Gene* 15:81), *tac* (Amann, E. et al. (1983) *Gene* 25:167), *trc* (Amann, E. et al. (1988) *Gene* 69:301), and promoters such as *lacUV5*, *lpp*, P_R, and hybrid and tandem promoters constructed to combine specific features to increase strength or regulation capacity (Balbas, P. and Bolivar, F. (1990) *Methods in Enzymology* 185:14).

Biochemical Evidence of Enzymatic Function

The enzyme *p*-hydroxyphenylpyruvate dioxygenase catalyzes the reaction of *p*-hydroxyphenylpyruvate with molecular oxygen to give homogentisate and CO_2. The enzyme can be assayed by measuring oxygen utilization (Hager, S. E., et al. (1957) *J. Biol. Chem.* 225:935-947), CO_2 release or homogentisate production from radioactive labeled *p*-hydroxyphenylpyruvate (Lindblad, B. (1971) *Clin. Chem. Acta* 34:113-121), loss of the *p*-hydroxyphenylpyruvate (Lin, E. C. C. et al. (1958) *J. Biol. Chem.* 233:668-673), or formation of homogentisate using a colorimetric assay (Fellman, J. H. et al. (1972) *Biochim. Biophys. Acta* 284:90-100) or UV detection following HPLC or a similar chromatographic separation technique. The activity of *p*-hydroxyphenylpyruvate dioxygenase may also be measured in a coupled assay in which the initial product, homogentisate, is oxidized by homogentisate dioxygenase; formation of maleylacetoacetate determined by measuring absorbance at 330 nm (Fernández-Cañón, J. M. and Peñalva, M. A. (1997) *Anal. Biochem.* 245:218-221).

An alternative to any of the kinetic assays for *p*-hydroxyphenylpyruvate dioxygenase is an end-point or fixed-time assay. The procedure is based on the conversion of unconverted substrate, *p*-hydroxyphenylpyruvate to its enediol tautomer by tautomerase in the presence of borate ions and measurement of the characteristic 308 nm peak of the tautomer (Lin, E. C. C. et al. (1958) *J. Biol.*

Chem. 233:668-673). The procedure involves the addition of enough *p*-hydroxyphenylpyruvate dioxygenase to consume ~80% of the organic substrate over 1 hour in 200 μL of assay buffer, which in this case is a 50 mM Tris, pH 7.4, 0.10 mM *p*-hydroxyphenylpyruvic acid, 1.75 mM ascorbate and 1.25 mM EDTA. After 1 hr the reaction is quenched by the addition of 100 μL of 0.8 M borate, pH 7.3, containing 1000 ppb of a *p*-hydroxyphenylpyruvate dioxygenase inhibitor and 0.25 μL of 6.1 mg/mL of tautomerase. The absorbance at 308 nm is read after a 30 min incubation and is stable thereafter for 2 hr. The advantage of this assay over the kinetic procedure is that the *p*-hydroxyphenylpyruvate dioxygenase is not required to oxidize the substrate in the presence of high concentrations of borate, a condition that might interfere with the mode of action of inhibitors. Furthermore the assay produces essentially a stable binary indication of *p*-hydroxypheny-lpyruvate dioxygenase inhibition, and is well-suited for applications which require a high-throughput of samples and assays.

The enzyme encoded by the nucleic acid fragments and overexpressed in *E. coli* can be extracted in any conventional buffer used for extracting soluble plant enzymes. Although a large amount of an overexpressed protein is often insoluble, the amount that is soluble represents can represent as much as 50% of the total soluble protein. Soluble overexpressed protein has high *p*-hydroxy-phenylpyruvate dioxygenase activity and is easily extracted. Likewise, it may be possible to resolubilize an insoluble overexpressed protein in an active form under appropriate conditions, since addition of sarkosyl (sodium N-lauroylsarcosinate) to the extraction buffer appeared to increase the amount of the overexpressed protein extracted. For optimum activity, a reducing agent such as ascorbate or reduced glutathione should be present as well as a source a ferrous ion.

An overexpressed enzyme can be assayed using all the techniques described above for measuring *p*-hydroxyphenylpyruvate dioxygenase activity, while only the techniques using labeled *p*-hydroxyphenylpyruvate can be used to measure activity in crude plant extracts. Therefore, the availability of an overexpressed enzyme greatly facilitates the development of high capacity screens to identify inhibitors of the enzyme. Potential inhibitors are evaluated for their capacity to reduce the rate of the reaction of the enzyme, resulting in reduced oxygen uptake and CO_2 release, and lower rates of formation of homogentisate and loss of *p*-hydroxyphenylpyruvate. Applicants have demonstrated that at least one of the instant nucleic acid fragments can be overexpressed in *E. coli* cells, resulting in production of a protein that catalyzes the conversion of *p*-hydroxy-phenylpyruvate to homogentisate with the release of CO_2. Furthermore, it has been shown that this activity is inhibited by commercial herbicides known to

inhibit *p*-hydroxyphenylpyruvate dioxygenase. Finally, an overexpressed enzyme can be used in a high capacity assay to identify compounds that inhibit the enzymatic activity of *p*-hydroxyphenylpyruvate dioxygenase. Such compounds may serve as herbicides.

<u>Preparation of Plants Tolerant to Inhibitors of *p*-Hydroxyphenylpyruvate Dioxygenase</u>

This invention embodies plants which are resistant or at least tolerant to herbicides that target the *p*-hydroxyphenylpyruvate dioxygenase enzyme at levels which are normally inhibitory to the naturally occurring *p*-hydroxyphenylpyruvate dioxygenase enzyme. This altered *p*-hydroxyphenylpyruvate dioxygenase activity is conferred by (1) overexpression of the wild-type *p*-hydroxyphenylpyruvate dioxygenase enzyme, or (2) expression of a DNA molecule encoding a herbicide-tolerant enzyme. The said enzyme may be a modified form of an *p*-hydroxyphenylpyruvate dioxygenase enzyme that occurs naturally in a eukaryote or prokaryote, or a modified form of an *p*-hydroxyphenylpyruvate dioxygenase enzyme that naturally occurs in a plant, or a herbicide tolerant enzyme that naturally occurs in a prokaryote (Duke et al. *Herbicide Resistant Crops*; Lewis: Boca Raton:1994). An effective amount of gene expression to render the cells of the plant tissue substantially tolerant to the herbicide depends on whether the gene codes for an unaltered *p*-hydroxyphenylpyruvate dioxygenase gene or a mutant or altered form of the gene that is less sensitive to the herbicides. Expression of an unaltered plant *p*-hydroxyphenylpyruvate dioxygenase gene in an effective amount is that amount that provides for a 2- to 10-fold increase in herbicide tolerance. Plants encompassed by the invention include monocotyledoneous and dicotyledoneous plants. Preferred are those plants which would be potential targets for *p*-hydroxyphenylpyruvate dioxygenase-inhibiting herbicides, particularly agronomically important crops such as maize and other cereal crops.

Increased levels of expression of *p*-hydroxyphenylpyruvate dioxygenase activity, from two to ten or more times the natively expressed amount, would be sufficient to overcome growth inhibition caused by the herbicide. Plants containing such altered *p*-hydroxyphenylpyruvate dioxygenase enzyme activity can be obtained by direct selection in plants. This method is known in the art. See, e.g., U.S. Patent No. 5,162,602, U.S. Patent No. 4,761,373, and references cited therein.

Overexpression of *p*-hydroxyphenylpyruvate dioxygenase also can be accomplished by stably transforming a host plant cell with a chimeric DNA molecule comprising a promoter capable of driving expression of an associated coding sequence in a plant cell and operably linked to a homologous or

heterologous coding sequence encoding *p*-hydroxyphenylpyruvate dioxygenase. A "homologous" *p*-hydroxyphenylpyruvate dioxygenase gene is isolated from an organism taxonomically identical to the target plant cell, whereas a "heterologous" *p*-hydroxyphenylpyruvate dioxygenase gene is obtained from an organism taxonomically distinct from the target plant.

The expression of foreign genes in plants is well-established (De Blaere et al., (1987) *Meth. Enzymol.* 143:277-291). Promoters utilized to drive gene expression in transgenic plants or plant cells (i.e., those capable of driving expression of the associated coding sequences such as *p*-hydroxyphenylpyruvate dioxygenase in plant cells, include those directing the 19S and 35S transcripts in Cauliflower mosaic virus (Odell et al., (1985) *Nature* 313:810-812; Hull et al., (1987) *Virology* 86:482-493), small subunit of ribulose 1,5-bisphosphate carboxylase (Morelli et al., (1985) *Nature* 315:200-204; Broglie et al., (1984) *Science* 224:838-843; Hererra-Estrella et al., (1984) *Nature* 310:115-120; Coruzzi et al.,.(1984) *EMBO J.* 3:1671-1679; Faciotti et al., (1985) *Bio/Technology* 3:241 and chlorophyll a/b binding protein (Lamppa et al., (1986) *Nature* 316:750-752); nopaline synthase promoters (Depicker et al. (1982) *J. Mol. App. Genet.* *1*:561-573; An et al. (1990) *Plant Cell* *2*:225-233). The chimeric DNA construct(s) of the invention may contain multiple copies of a promoter or multiple copies of the *p*-hydroxyphenylpyruvate dioxygenase coding sequences. In addition, the construct(s) may include coding sequences for selectable markers and coding sequences for other peptides such as signal or transit peptides. The preparation of such constructs is within the ordinary level of skill in the art. Resistance to inhibitors of the plant carotenoid biosynthesis pathway, which is also targeted by *p*-hydroxyphenylpyruvate dioxygenase inhibitors, has been achieved by expressing a bacterial gene encoding phytoene desaturase driven by the CaMV promoter (Misawa et al., (1994) *Plant. J.* *4*:481-490).

Transit peptides may be fused to the *p*-hydroxyphenylpyruvate dioxygenase coding sequence in the chimeric DNA constructs of the invention to direct transport of the expressed *p*-hydroxyphenylpyruvate dioxygenase enzyme to the desired site of action. Examples of transit peptides include the chloroplast transit peptides such as those described in Von Heijne et al., (1991) *Plant Mol. Biol. Rep.* 9:104-126; Mazur et al., (1987) *Plant Physiol.* 85:1110; Vorst et al., (1988) *Gene* 65:59; and mitochondrial transit peptides such as those described in Boutry et al., (1987) *Nature* 328:340-342.

It is envisioned that the introduction of enhancers or enhancer-like elements into other promoter constructs will also provide increased levels of primary transcription to accomplish the invention. These would include viral enhancers

such as that found in the 35S promoter (Odell et al., (1988) *Plant Mol. Biol.* 10:263-272), enhancers from the opine genes (Fromm et al., (1989) *Plant Cell* 1:977-984), or enhancers from any other source that result in increased transcription when placed into a promoter operably linked to the nucleic acid fragment of the invention.

Introns isolated from the maize Adh-1 and Bz-1 genes (Callis et al., (1987) *Genes Dev.* 1:1183-1200), and intron 1 and exon 1 of the maize Shrunken-1 (sh-1) gene (Maas et al., (1991) *Plant Mol. Biol.* 16:199-207) may also be of use to increase expression of introduced genes. Results with the first intron of the maize alcohol dehydrogenase (Adh-1) gene indicate that when this DNA element is placed within the transcriptional unit of a heterologous gene, mRNA levels can be increased by 6.7-fold over normal levels. Similar levels of intron enhancement have been observed using intron 3 of a maize actin gene (Luehrsen. K. R. and Walbot, V., (1991) *Mol. Gen. Genet.* 225:81-93). Enhancement of gene expression by Adh1 intron 6 (Oard et al., (1989) *Plant Cell Rep* 8:156-160) has also been noted. Exon 1 and intron 1 of the maize sh-1 gene have been shown to individually increase expression of reporter genes in maize suspension cultures by 10 and 100-fold, respectively. When used in combination, these elements have been shown to produce up to 1000-fold stimulation of reporter gene expression (Maas et al., (1991) *Plant Mol. Biol.* 16:199-207).

Any 3' non-coding region capable of providing a polyadenylation signal and other regulatory sequences that may be required for proper expression can be used to accomplish the invention. This would include the 3' end from any storage protein such as the 3' end of the 10kd, 15kd, 27kd and alpha zein genes, the 3' end of the bean phaseolin gene, the 3' end of the soybean β-conglycinin gene, the 3' end from viral genes such as the 3' end of the 35S or the 19S cauliflower mosaic virus transcripts, the 3' end from the opine synthesis genes, the 3' ends of ribulose 1,5-bisphosphate carboxylase or chlorophyll a/b binding protein, or 3' end sequences from any source such that the sequence employed provides the necessary regulatory information within its nucleic acid sequence to result in the proper expression of the promoter/coding region combination to which it is operably linked. There are numerous examples in the art that teach the usefulness of different 3' non-coding regions (for example, see Ingelbrecht et al., (1989) *Plant Cell* 1:671-680).

Various methods of introducing a DNA sequence (i.e., of transforming) into eukaryotic cells of higher plants are available to those skilled in the art (see EPO publications 0 295 959 A2 and 0 138 341 A1). Such methods include high-velocity ballistic bombardment with metal particles coated with the nucleic acid

constructs (see Klein et al., (1987) *Nature* (London) 327:70-73. and see U.S. Patent No. 4,945,050), as well as those based on transformation vectors based on the Ti and Ri plasmids of *Agrobacterium* spp., particularly the binary type of these vectors. Ti-derived vectors transform a wide variety of higher plants, including monocotyledonous and dicotyledonous plants, such as soybean, cotton and rape seed (Pacciotti et al., (1985) *Bio/Technology* 3:241; Byrne et al., (1987) *Plant Cell, Tissue and Organ Culture* 8:3; Sukhapinda et al., (1987) *Plant Mol. Biol.* 8:209-216; Lorz et al., (1985) *Mol. Gen. Genet.* 199:178-182; Potrykus et al., (1985) *Mol. Gen. Genet.* 199:183-188).

Other transformation methods are available to those skilled in the art, such as direct uptake of foreign DNA constructs (see EPO publication 0 295 959 A2), and techniques of electroporation (see Fromm et al., (1986) *Nature* (London) 319:791-793). Once transformed, the cells can be regenerated by those skilled in the art. Also relevant are several recently described methods of introducing nucleic acid fragments into commercially important crops, such as rapeseed (see De Block et al., (1989) *Plant Physiol.* 91:694-701), sunflower (Everett et al., (1987) *Bio/Technology* 5:1201-1204), soybean (McCabe et al., (1988) *Bio/Technology* 6:923-926; Hinchee et al., (1988) *Bio/Technology* 6:915-922; Chee et al., (1989) *Plant Physiol.* 91:1212-1218; Christou et al., (1989) *Proc. Natl. Acad. Sci USA* 86:7500-7504; EPO Publication 0 301 749 A2), and corn (Gordon-Kamm et al., (1990) *Plant Cell* 2:603-618; and Fromm et al., (1990) *Bio/Technology* 8:833-839).

Altered *p*-hydroxyphenylpyruvate dioxygenase enzyme activity may also be achieved through the generation or identification of modified forms of the isolated eukaryotic *p*-hydroxyphenylpyruvate dioxygenase coding sequence having at least one amino acid substitution, addition or deletion which encodes an altered *p*-hydroxyphenylpyruvate dioxygenase enzyme resistant to a herbicide that inhibits the unaltered, naturally occurring form. Genes encoding such enzymes can be obtained by numerous strategies known in the art. A first general strategy involves direct or indirect mutagenesis procedures on microbes (e.g., *E. coli*, *S. cerevisiae* (Miller, (1972) Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Davis et al., (1980) Advanced Bacterial Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY; Sherman et al., (1983) Methods in Yeast Genetics, Cold Spring Harbor Laboratory, Gold Spring Harbor NY; and U.S. Patent No. 4,975,374) and cyanobacteria (Bryant, *The Molecular Biology of Cyanobacteria*; Kluwer Academic Publishers: Boston, 1995). A second method of obtaining mutant herbicide-resistant alleles of the eukaryotic *p*-hydroxyphenylpyruvate dioxygenase

enzyme involves direct selection in plants. For example, the effect of inhibitors on the growth of plants such as Arabidopsis, soybean, or maize may be determined by plating seeds sterilized by art-recognized methods on plates on a simple minimal salts medium containing increasing concentrations of the inhibitor. The lowest dose at which significant growth inhibition can be reproducibly detected is used for subsequent experiments. Mutagenesis of plant material may be utilized to increase the frequency at which resistant alleles occur in the selected population. Mutagenized seed material can be derived from a variety of sources, including chemical or physical mutagenesis or seeds, or chemical or physical mutagenesis or pollen (Neuffer, In Maize for Biological Research. Sheridan, ed. Univ. Press, Grand Forks, ND., pp. 61-64 (1982)), which is then used to fertilize plants and the resulting M1 mutant seeds collected. Typically, for Arabidopsis, M2 seeds (i.e., progeny seeds of plants grown from seeds mutagenized with chemicals, such as ethyl methane sulfonate, or with physical agents, such as gamma rays or fast neutrons) are plated at densities of up to 10,000 seeds/plate (10 cm diameter) on minimal salts medium containing an appropriate concentration of inhibitor. Seedlings that continue to grow and remain green 7-21 days after plating are transplanted to soil and grown to maturity and seed set. Progeny of these seeds are tested for resistance to the herbicide. If the resistance trait is dominant, plants whose seed segregate 3:1 (resistant:sensitive) are presumed to have been heterozygous for the resistance at the M2 generation. Plants that give rise to all resistant seed are presumed to have been homozygous for the resistance at the M2 generation. Such mutagenesis on intact seeds and screening of their M2 progeny seed can also be carried out on other species, for instance soybean (see, e.g., U.S. Patent No. 5,084,082). Mutant seeds to be screened for herbicide tolerance can also be obtained as a result of fertilization with pollen mutagenized by chemical or physical means.

EXAMPLE 1

Cloning of a cDNA for *Arabidopsis thaliana* *p*-Hydroxyphenylpyruvate Dioxygenase

The plasmid containing the *Arabidopsis thaliana* 91B13T7 expressed sequence tag (Newman et al., (1994) *Plant Physiol* 106:1241-1255) was digested with the restriction enzymes *Bam*HI and *Eco*RI, and the resulting 400 bp fragment was used to screen a lambda phage cDNA library of *Arabidopsis thaliana* seedlings (Scolnik, P. A. and Bartley, G. E. (1994) *Plant Physiol.* 104:1469-1470) according to the following protocol.

E. coli KW251 cells were grown overnight in Luria Broth ("LB") containing 0.2% maltose and 10 mM $MgSO_4$. Cells were pelleted by centrifugation and

resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5. Cell aliquots (0.8 mL) were mixed with 0.1 mL of diluted phage samples and 7 mL of top agarose (0.7% agarose in LB containing 10 mM $MgSO_4$) at 45°C, and plated onto 150 mm Petri dishes containing LB agar. Phage plaques became visible in 5-7 h, at which point the plates were placed at 4°C.

Phage plaques were transferred to nitrocellulose filters according to standard techniques, and the filters were hybrized to ^{32}P-radiolabeled probe prepared according to the method of Feinberg and Vogelstein ((1983) *Anal. Biochem.* 132:6-13), using the hybridization conditions of Berlyn et al.((1989) *Proc. Natl. Acad. Sci.* 86:4604-4608). After exposure to X-ray film for 48 h, 12 positive plaques were eluted, plated, and hybridized under the same conditions. A total of 9 plaques that retained positive signals in this second round of hybridization were subjected to *in vivo* excision using the Exassist/SOLR™ system according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). DNA from the plasmids resulting from *in vivo* excision of positive plaques was prepared for DNA sequencing using the Wizard Plus™ kit (Promega, Madison, WI). Eight of the clones that were sequenced showed strong conservation with available *p*-hydroxyphenylpyruvate dioxygenase sequences, whereas the remaining clone did not correspond to a *p*-hydroxyphenylpyruvate dioxygenase. Alignment with known *p*-hydroxyphenylpyruvate dioxygenase sequences also revealed that two of the clones correspond to 0.3 kbp fragments from the 3' end of the transcript, and another two to 1.2 kbp fragments from the 5' end of the transcript. One clone of each was used to assemble a 1.5 kbp cDNA by ligating at the internal *Nhe*I restriction site (Figure 1). The initial determination of the DNA sequence (SEQ ID NO:2) of the resulting cDNA clone is shown in Figure 2. Subsequent work with this DNA fragment required confirmation of some of the features of its sequence. Approximately ten nucleotide residues were found to have been listed in error. Thus a corrected sequence for this DNA fragment is listed in SEQ ID NO:14 and the deduced amino acid sequence is set forth in SEQ ID NO:15. The revised sequences form the bases for analyses and comparisons reported herein.

EXAMPLE 2

Overexpression of the *Arabidopsis* cDNA in *E. coli*

The deduced amino acid sequence for *Arabidopsis p*-hydroxyphenylpyruvate dioxygenase was aligned with the amino acid sequences of *p*-hydroxyphenylpyruvate dioxygenase from mouse, pig, and *Streptomyces avermitilis* using the Pileup program of GCG (Program Manual for the Wisconsin Package, Version 8, September 1994, Genetics Computer Group, 575 Science Drive, Madison, WI, USA 53711). This analysis suggested an additional

29 amino acid-extension at the amino terminus of the *Arabidopsis* sequence (positions 1-29, Figure 3 and SEQ ID NO:3). This amino-terminal extension was assumed to be a chloroplast transit peptide which would be absent from the mature enzyme. Therefore, removal of the chloroplast transit peptide coding sequence coincided with transfer of the *p*-hydroxyphenylpyruvate dioxygenase coding sequence from the cloning vector into the expression vector.

The *Arabidopsis p*-hydroxyphenylpyruvate dioxygenase cDNA was moved from the pBluescript SK- cloning vector (Stratagene, La Jolla, CA) to the pET24c(+) expression vector (Novagen, Madison, WI) through the intermediate cloning vector pT7BlueR (Novagen). The plasmid pGBPPD2 consists of the *Arabidopsis p*-hydroxyphenylpyruvate dioxygenase cDNA and the pBluescript SK- cloning vector (Stratagene). The plasmid pE24CP1 consists of the *Arabidopsis p*-hydroxyphenylpyruvate dioxygenase cDNA, without the putative chloroplast transit peptide DNA sequence, and the pET24c(+) expression vector (Novagen).

The plasmids pGBPPD2 and pT7BlueR (5 μg each) were individually digested with 20 units of Xba I (New England Biolabs, NEB, Beverly, MA) and 20 units of Hind III (Gibco BRL, Gaithersburg, MD) in NEB restriction enzyme buffer 2 supplemented with 100 μg/mL bovine serum albumin at 37 °C for 1.75 h. Digesting pGBPPD2 with the restriction enzymes Xba I and Hind III releases the 5' and 3' ends, respectively, of the *p*-hydroxyphenylpyruvate dioxygenase cDNA from the pBluescript SK- polylinker. Products of the digestion were electrophoretically separated in a 1 percent agarose gel using TRIS/acetate/EDTA (TAE) buffer and visualized with ethidium bromide staining (Maniatis). Digestion of pGBPPD2 with the two restriction endonucleases resulted in a 2922 bp vector band and 1499 bp *p*-hydroxyphenylpyruvate dioxygenase cDNA band. Only a 2863 bp band was apparent after digesting pT7BlueR with the two enzymes, although a 24 bp fragment would also result. The 1499 bp *p*-hydroxyphenylpyruvate dioxygenase band and the 2863 bp T7BlueR band were cut out of the gel and the associated DNA purified from the agarose using a QIAquick Gel Extraction Kit (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. The purified DNA samples were precipitated by the addition of sodium acetate (pH 5.2) to 0.3 M, 10 μg tRNA (added as carrier), two volumes of -20 °C ethanol and incubation at -20 °C overnight. Nucleic acid pellets were collected by centrifugation, washed with 70% ethanol and air dried. Both pellets were solublized in 10 μL of TRIS/EDTA (TE) buffer, pH 8 (Maniatis), and then 1 μL of each sample loaded onto a 1% agarose, TAE gel in separate wells next to a well containing 4 μL of Mass Ladder (Gibco BRL). All samples were adjusted

to 10 μL with water before loading. DNA was quantified by comparing band intensities of each sample with Mass Ladder band intensities following ethidium bromide staining and UV illumination.

Approximately 300 ng of *p*-hydroxyphenylpyruvate dioxygenase insert was mixed with 300 ng of double digested pT7BlueR vector in a total volume of 7 μL and then heated to 45 °C for 5 min followed by cooling on ice. T4 DNA ligase buffer (Gibco BRL) and 1 unit of T4 DNA ligase (Gibco BRL) were added to the cooled DNA for a total volume of 10 μL. The ligation mix was incubated at room temperature for 4 h and then transformed into MAX Efficiency DH5α Competent Cells (Gibco BRL) of *E. coli* according to standard procedures (Maniatis). Transformed bacteria were spread onto LB agar plates supplemented with 100 μg/mL carbenicillin and incubated overnight at 37 °C. Seventeen bacterial colonies were selected for subsequent analysis. A portion of each colony was inoculated into a separate 17x100 mm polypropylene culture tube (Falcon, Lincoln Park, NJ) containing 2 mL of liquid LB media and 200 μg/mL carbenicillin. Liquid bacteria cultures were incubated overnight at 37 °C with shaking (250 rpm). Plasmid DNA was then isolated using a QIAprep Spin Plasmid Miniprep Kit (Qiagen) according to the manufacturer's instructions. A portion (5 μL out of 50 μL total) of each plasmid preparation was digested with 10 units each of Hind III and EcoR V (Gibco BRL) in a total volume of 15 μL with React 2 buffer (Gibco BRL) for one h. (Note: The EcoRV site in the pBluescript polylinker was destroyed during the preparation of pGBPPD2 so only the EcoRV site in the pT7BlueR polylinker would be accessible to the restriction nuclease). Samples were separated electrophoretically in 1% agarose and tris/borate/EDTA (TBE) buffer (Maniatis). Bands were visualized with ethidium bromide staining; 7 out of 17 samples which contained 2 bands (2837 and 1525 bp) contained the *p*-hydroxyphenylpyruvate dioxygenase insert and were designated pT7BlueR+PDO1 (see Figure 4).

In order to remove the putative chloroplast transit sequence, the remaining 45 μL of each prep of pT7BlueR+PDO1 were combined into a single sample and the DNA content determined spectrophotometrically at A_{260} (Maniatis). A portion (5 μg) of pT7BlueR+PDO1 was digested with 16 units of Eco47 III (MBI Fermentas) in a total volume of 100 μL containing buffer 0 (MBI Fermentas) at 37 °C for 2 h. The digested plasmid DNA was then precipitated with sodium acetate and ethanol as above and the resulting dried nucleic acid pellet was dissolved in 60 μL of React 2 (Gibco BRL) containing 20 units of Nde I (Gibco BRL) and incubated 2 h at 37 °C. The double digested sample was then loaded onto a 1% agarose gel in TAE and the large 4166 bp Nde I-Eco47III fragment

separated from the 196 bp fragment electrophoretically. The large fragment was cut out of the gel, purified from agarose and precipitated as above.

An oligonucleotide mix was prepared consisting of 100 pmoles each of oligos CAM32 and CAM33 (SEQ ID NOS:4 and 5, respectively) in a combined volume of 9.9 μL. The two oligos complement each other to form a 3' blunt end corresponding to the 5' half of an Eco47 III restriction site and also form a 5' staggered end which corresponds to the 3' half of an Nde I restriction site.

CAM 32: (SEQ ID NO:4)
5'-TATGTCCAAGTTCGTAAGAAAGAATCCAAAGTCTGATAAATTCAAGGTTAAGC-3'

CAM 33: (SEQ ID NO:5)
5'-GCTTAACCTTGAATTTATCAGACTTTGGATTCTTTCTTACGAACTTGGACA-3'

The oligo mix was heated to 90 °C for 1.5 min and then allowed to cool to room temperature over 20 min. The dried nucleic acid pellet resulting from purification of the 4166 bp Nde I-Eco47 III fragment was solublized in 7 μL of the cooled oligo mix and subsequently heated to 45 °C for 5 min followed by cooling on ice. Ligation of the oligos with the Nde I-Eco47 III fragment followed by transformation into DH5α was performed as above. Transformed bacterial cells were spread onto LB/carbenicillin plates and incubated at 37 °C overnight. Seventeen colonies were selected and processed to isolate plasmid DNA as above. A portion (5 out of 50 μL) of each plasmid was double digested with 10 units each of Nde I and Hind III and the fragments separated electrophoretically on a 1% agarose gel in TBE. A two band pattern corresponding to insert (1373 or 1518 bp) and vector (2844 bp) was detected. An additional double digest with 10 units each of Xba I and Hind III was performed on another 5 μL aliquot of plasmids. When digested with Nde I and Hind III, none of the plasmids which contained the smaller insert size contained a Xba I site. The Xba I site would be eliminated if the two oligos replaced the 196 bp fragment originally present in pT7Blue+PDO1. The 7 plasmid samples with the modified *p*-hydroxyphenylpyruvate dioxygenase insert were combined and designated pT7BlueR+PDO2.

The pT7BlueR+PDO2 plasmid DNA was quantified spectrophotometrically (above) and then 5 μg was digested with 20 units each of Hind III and Nde I in 62 μL of React 2 for 2 h at 37 °C. The digested sample was subsequently loaded onto a 1% agarose gel in TAE and separated electrophoretically. The 1373 bp fragment was isolated and precipitated as above. The plasmid pET24c(+) (5 μg) was double digested with 20 units each of both Nde I and Hind III in React 2 at 37 °C for 2 h and the 5245 bp fragment then gel purified on a 1% agarose gel in

TAE and subsequently separated from agarose and precipitated as above. The dried pET24c(+) pellet was solublized in 10 μL TE and then 8 μL was adjusted to a 20 μL total volume with water, dephosphorylation buffer (Gibco BRL) and 1 unit of calf intestinal alkaline phosphatase (Gibco BRL). The sample was incubated at 37 °C for 30 min and then gel purified, separated from agarose, and precipitated as above. The dried, dephosphorylated, pET24c(+) vector pellet and modified *p*-hydroxyphenylpyruvate dioxygenase insert pellet were each solublized in 10 μL TE and then 1 μL of each was run on a 1% agarose TBE gel with 4 μL of mass ladder to quantify DNA as above. One hundred nanograms of modified *p*-hydroxyphenylpyruvate dioxygenase insert was mixed with 120 ng of dephosphorylated pET24c(+) vector in a total of 7 μL volume. The mix was heated to 45 °C for 5 min and then cooled on ice. The mix was then supplemented with T4 DNA ligase buffer and 1 unit of T4 DNA ligase in a total volume of 10 μL and the mix allowed to incubate at room temperature for 4 h. The ligation mix was subsequently transformed into DH5α, spread on LB agar supplemented with 30 μg/mL kanamycin, and incubated overnight at 37 °C. Plasmid preparations were performed on 11 colonies as above. Plasmids were double digested with Nde I and Hind III and fragments separated electrophoretically. All plasmids had the expected 1373 bp and 5245 bp fragments. One bacteria colony was selected and used to inoculate 100 mL of liquid LB supplemented with 30 μg/mL kanamycin which was subsequently incubated at 37 °C overnight with shaking. Plasmid DNA was isolated from the resulting bacteria culture using a Qiagen Plasmid Midi Kit according to the manufacturer's instructions. A portion of the plasmid DNA (pE24CP1) was sequenced with the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical, Cleveland, OH) using a biotinylated sequencing primer to the T7 promoter (United State Biochemical) according to the manufacturer's instructions for non-radioactive manual sequencing. DNA was transferred from the sequencing gel to Hybond-N+ nylon transfer membrane (Amersham, Arlington Heights, IL) by capillary action. Transfer and all subsequent steps in chemiluminescent detection of DNA fragments were performed with a SEQ-Light Chemiluminescent Sequencing System kit (Tropix, Bedford, MA) according to the manufacturer's instructions. DNA sequencing verified that the plasmid contained the expected 5' sequence for the modified *p*-hydroxyphenylpyruvate dioxygenase insert where nucleotides 1-95 (Figure 2) were replaced with an ATG transcriptional start site. This is equivalent to amino acids 2-29 (Figure 3) being eliminated from the N-terminus of the *Arabidopsis p*-hydroxyphenylpyruvate dioxygenase amino acid sequence.

The plasmid pE24CP1 was transformed into competent cells of BL21(DE3) *E. coli* (Novagen), as above. Transformed cells were spread on LB/kanamycin plates and incubated overnight at 37 °C. Seven colonies were selected for plasmid preparations as above and plasmid DNA was double digested with Nde I and Hind III to verify that all plasmids had the expected electrophoretic banding pattern. One colony was selected and streaked for isolation on LB/kanamycin plates. A well isolated colony was used to inoculate liquid LB supplemented with 30 μg/mL kanamycin and the culture was incubated at 37 °C with shaking (250 rpm) until it reached an A_{600} of 0.6 absorbance units. An 8% glycerol freezer stock was prepared according to the Novagen protocol and stored at -80 °C. All subsequent expression studies were done with freshly grown bacterial cells that were isolated from LB/kanamycin plates streaked from the glycerol freezer stock.

BL21(DE3) *E. coli* cells containing either pE24CP1 or pET24c(+) (negative control) were streaked out onto LB/kanamycin plates from a glycerol freezer stock (above) and incubated overnight at 37 °C. One isolated colony was selected for inoculation of 2 mL of LB containing 30 μg/mL kanamycin in a 17 x 100 mm Falcon tube, and the culture was incubated at 37 °C with shaking (250 rpm) overnight. The overnight cultures were then used to inoculate 100 mL of fresh LB containing 30 μg/mL kanamycin. The new cultures were incubated at 37 °C with shaking until the A_{600} reached between 0.4 and 0.6 absorbance units. One half of the pE24CP1 and pET24c(+) cultures were placed in new culture flasks and IPTG (isopropylthio-β-D-galactoside; Gibco BRL) was added to the new flasks to give a final concentration of 1 mM. The flasks were incubated an additional 3 h at 37 °C with shaking, and then the cells were harvested.

The harvested cells were centrifuged and the resulting cell pellet extracted by sonication (3 x 10 sec bursts) in 2 mL extraction buffer (50 mM (20 mM in the first experiment; Table 2) potassium phosphate buffer, pH 7.2, containing 0.14 M KCl, 0.32 mM reduced glutathione, 1% polyvinylpolypyrrolidone, and 0.1% Triton X 100 (0.01% lysozyme was included in the first experiment only)). The lysate represents the crude extracted enzyme after centrifugation at 17000 g for 10 min. In the first experiment (Table 2) a 20 to 60% ammonium sulfate precipitated enzyme fraction was also assayed. Solid ammonium sulfate was slowly added with stirring to 2 mL of the lysate to bring the concentration to 20% (w/v). After incubation on ice for approximately 15 min, the solution was centrifuged at 17000 g for 10 min. The supernatant liquid was harvested and solid ammonium sulfate was added to increase the concentration to 60% (w/v). After

centrifugation, the resulting pellet was resuspended in 1 mL of the extraction buffer.

A portion of the insoluble protein resulting from expression of *Arabidopsis* *p*-hydroxyphenylpyruvate dioxygenase in bacteria was utilized for N-terminal sequence analysis. The protein (approximately 180 μg) was suspended in 60 μL of extraction buffer and then diluted with 5 volumes of sample buffer (62.5 mM Tris, pH 6.8, 6 M urea, 160 mM dithiothreitol, 0.01% bromophenol blue) followed by intermittent vortexing for one hour at room temperature. A 1.5 mm thick, 12% polyacrylamide resolving gel was prepared for a Mini-Protein II dual slab cell (Bio-Rad, Hercules, CA) using the manufacturer's instructions. The polyacrylamide was allowed to polymerize for 3 h and then a stacking gel was prepared using a preparative comb. The running buffer was prepared according to the manufacturer's instructions with the addition of 0.1 mM sodium thioglycolate. The solublized protein sample was electrophoretically separated using the manufacturer's instructions. When the bromophenol blue dye front reached the bottom of the gel, the gel was removed and equilibrated for 5 min in blotting buffer (10 mM CAPS, pH 11, 10% methanol, balance water). The gel was then placed in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad), according to the manufacturer's instructions, with a ProBlott PVDF membrane (Applied Biosystems, Foster City, CA) treated according to the manufacturer's instruction. Electroblotting was done in the presence of blotting buffer at 50 volts for 45 min in an ice bath. The membrane was then rinsed in water and stained with Coomassie Blue as described in the ProBlott protocol. The major protein band was excised from the membrane and subjected to N-terminal amino acid sequencing on a Beckman (Fullerton, CA) LF3000 protein sequencer. The first 11 cycles identified S-K-F-V-R-K-N-P-K-S-D (see SEQ ID NO:3, amino acids 30-40), respectively. This is the expected N-terminus of the modified *Arabidopsis* *p*-hydroxyphenylpyruvate dioxygenase minus the initial methionine (amino acids 30-40, Figure 3).

EXAMPLE 3

p-Hydroxyphenylpyruvate Dioxygenase Enzymatic Activity of the Plant Protein Expressed in *E. Coli*

Cell cultures with different plasmid constructs were extracted as described above and assayed by measuring the formation of $^{14}CO_2$ from [1-^{14}C]-*p*-hydroxyphenylpyruvate or $^{14}CO_2$ and ^{14}C-homogentisate from [U-^{14}C]-*p*-hydroxyphenylpyruvate (Lindblad, B., (1971) *Clin. Chim. Acta* 34:113-121; and Lindstedt, S. and Odelhog, B., (1987) *Methods in Enzymology* 142:143-148). The labeled substrate was prepared from [1-^{14}C]-L-tyrosine

(55 mCi/mmol; American Radiolabeled Chemicals, Inc., St. Louis, MO) or [U-^{14}C]-L-tyrosine (498 mCi/mmol; DuPont NEN, Boston, MA). A 50-100 μL aliquot (5-10 μCi) of the of the labeled tyrosine stock solution was transferred to a 4 mL glass vial and blown to dryness in a stream of nitrogen at 45°C. To the vial was added 175 μL of 0.1 M phosphate buffer, pH 6.5, 5 μL catalase (28,700 units of C-100, Sigma Chemical Co., St. Louis, MO), and 20 μL L-amino acid oxidase (Sigma A-9253, 6.5 units/mL). The vial was then placed on a shaker water bath set at 30°C, 60 cycles/min, for 0.5 to 1 h. The reaction mix was then passed through a small column containing 400 μL Dowex AG 50W X8 cation exchange resin. The column was then washed with 1.5 mL of water and the eluant containing the labeled *p*-hydroxyphenylpyruvate was collected. The labeled substrate was either used immediately or stored at -80°C and used within a week after preparation.

The assay was performed in 14 mL culture tubes capped with serum stoppers through which a polypropylene well containing 200 μL of 1 N KOH was suspended. The reaction mixture contained 5,740 units of catalase, 100 μL of a freshly prepared 1:1 (v:v) mixture of 150 mM reduced glutathione and 3 mM dichlorophenolindophenol, 5 mM ascorbate, 0.1 mM ferrous sulfate (the ascorbate and ferrous sulfate were not present in the buffer used in the first experiment; Table 2), 50 μM unlabeled *p*-hydroxyphenylpyruvate, 1-25 μL of the enzyme extract, and 50 mM potassium phosphate buffer in a final volume of 980 μL. Unlabeled substrate was made fresh daily in 50 mM potassium phosphate buffer and allowed to equilibrate for at least 2 h at room temperature to insure that greater than 95% was in the keto form. The tubes were incubated for 10 min at 30°C in a shaking water bath prior to adding 20 μL (0.04 μCi) of ^{14}C-*p*-hydroxyphenylpyruvate. The reaction was terminated after 60 min by injecting 500 μl of 1 N sulfuric acid through the serum stopper. The vials were left on the shaker for another 30 min to insure complete capture of the released $^{14}CO_2$. The serum caps were then removed and the wells cut and dropped into 8 mL scintillation vials. Six mL of Formula-989 scintillation fluid (Packard Insturments, Meriden, CT) was added to the vials and the ^{14}C radioactivity was determined by scintillation counting. Table 2 summarizes the results of this experiment.

Table 2

p-Hydroxyphenylpyruvate Dioxygenase Activity of Extracts from *E. coli* Containing Different Plasmid Constructs

Plasmid	Inducer (1 mM IPTG)	Lysate		Ammonium Sulfate Precipitate	
		dpm * /mg	nmol/min x mg	dpm * /mg	nmol/min x mg
pET24c(+)	-	12,318	0.09	0	0.00
pET24c(+)	+	35,115	0.25	3,393	0.03
pE24CP1	-	24,607	0.17	126,761	0.89
pE24CP1	+	243,801	1.71	1,371,823	9.64

* ^{14}C : ^{12}C = 1 : 50; sp. act. of ^{14}C-*p*-hydroxyphenylpyruvate = 55 mCi/mmol

The results show there was little or no *p*-hydroxyphenylpyruvate dioxygenase activity in any of the cell cultures that did not have the plasmid containing the nucleic acid fragment encoding *p*-hydroxyphenylpyruvate dioxygenase (pET24c(+)) and the inducer of gene expression (IPTG). The gene and inducer together resulted in a marked increase in activity.

In the experiment with [U-^{14}C] *p*-hydroxyphenylpyruvate ("HPPA"), where both $^{14}CO_2$ and ^{14}C-homogentisic acid were measured, the reaction was initiated by adding 50 μL of labeled substrate (0.3 μCi) and was terminated with 100 μL of 10% phosphoric acid. The $^{14}CO_2$ released was determined by scintillation counting, while the level of homogentisic acid was determined by HPLC on a Zorbax RX-C8 column (4.6 x 250 mm) with an in-line radioactivity detector. Aliquots of 1.7 to 15 μL were taken from the reaction mix after centrifugation and diluted into the column equilibration buffer prior to injection. Separation was performed at ambient temperature with a flow rate of 1.0 mL/min and the following gradient with solvent A and B being water and methanol, each with 1% phosphoric acid: 0-2 min, isocratic at 95% A and 5% B; 2-17 min, linear gradient from 95 to 75% A and 5 to 25% B; 17-19 min linear gradient from 75 to 5% A and 25 to 95% B; 19-22 min, isocratic at 5% A and 95% B; 22-24 min, linear gradient from 5% to 95% A and 95 to 5% B. In this system homogentisate eluted at 10.8 min. The results from this experiment are shown in Table 3.

Table 3

p-Hydroxyphenylpyruvate Dioxygenase Activity of Cell Extracts Determined by CO_2Release and Homogentisic Acid Synthesis from [U-^{14}C] *p*-Hydroxyphenylpyruvate

Plasmid	Inducer (1 mM IPTG)	nmol/min x mg* $^{14}CO_2$	nmol/min x mg* Homogentisic acid
pET24c(+)	-	0.00	0.00
pET24c(+)	+	0.19	0.00
pE24CP1	-	4.68	4.76
pE24CP1	+	29.12	29.82

* $^{14}C : ^{12}C$ = 1 : 87.7; sp. act. of ^{14}C[U]-*p*-HPPA = 498 mCi /mmol

There was a tight correlation between the results from the assays of the two products of the reaction. The results confirmed there was no significant *p*-hydroxyphenylpyruvate dioxygenase activity in either cell culture that did not contain the nucleic acid fragment encoding *p*-hydroxyphenylpyruvate dioxygenase. There was measureable enzyme activity in the absence of the inducer, but when the inducer was added the activity increased greater than six-fold over uninduced cultures. These results and those of Table 2 clearly show that the nucleic acid fragment isolated and overexpressed in *E. coli* cells encodes a protein that catalyzes the conversion of *p*-hydroxyphenylpyruvate to homogentisate with the release of CO_2.

The overexpressed protein was also assayed spectrophotometrically at ambient temperature using the enol borate-tautomerase assay (Lin. E. C. C. et al., (1958) *J. Biol. Chem.* 233:668-673). The assay buffer contained 0.4 M borate (adjusted to pH 7.2 with 0.2 M sodium borate), 4 mM ascorbate, 2.5 mM EDTA, 40 μM *p*-hydroxyphenylpyruvate, and 0.5 units of tautomerase (Sigma T-6004) per 10 mL buffer. The reaction mix was used when the tautomerization of the substrate was complete (when absorbance at 308 nm had stabilized). The assay was initiated by adding 40 μL of the cell extracts to 960 μL of the assay buffer, and the reaction was followed by measuring the decrease in absorbance at 308 nm. Table 4 summarizes the results with extracts of the same four cell cultures described in Table 3.

Table 4

Spectrophotometric Assay of *p*-Hydroxyphenylpyruvate Dioxygenase Activity of Cell Extracts

Plasmid	Inducer (1 mM IPTG)	nmol *p*-HP lost/min x mg*
pET24c(+)	-	1.58
pET24c(+)	+	2.73
pE24CP1	-	4.91
pE24CP1	+	22.32

* Loss of *p*-hydroxyphenylpyruvate based on a molar extinction coefficient for the equilibrium mixture of 9850 as reported by Lin et al. ((1958) *J. Biol. Chem.* *233*: 668-673).

EXAMPLE 4

Inhibition of *p*-Hydroxyphenylpyruvate Dioxygenase by Commercial Herbicides

The enzymatic activity of the overexpressed protein is inhibited by two herbicides known to inhibit plant *p*-hydroxyphenylpyruvate dioxygenase: Sulcotrione (2-(2-chloro-4-methanesulfonylbenzoyl)-1,3-cyclohexanedione); and Isoxaflutole (5-cyclopropylisoxazol-4-yl 2-mesyl-4-trifluoromethylphenyl ketone). These two compounds were tested against the overexpressed protein using both the $^{14}CO_2$ and the continuous spectrophotometric enol borate-tautomerase assays. Both compounds were added to the assay buffers in 10 μL of acetone or dimethyl sulfoxide. The I_{50} values (concentration inhibiting the enzyme 50%) were calculated based on the percent inhibition observed over several concentrations of the inhibitor. The results of the assays are shown in Table 5.

Table 5

I_{50} Values of Inhibitors of Plant *p*-Hydroxyphenylpyruvate Dioxygenase

Compound	I_{50} value (nM) derived from $^{14}CO_2$ assay	spectrophotometric assay
sulcotrione	43	44
isoxaflutole	409	1042

These results clearly show that the *p*-hydroxyphenylpyruvate dioxygenase activity of the overexpressed protein is inhibited by commercial herbicides that have inhibition of this enzyme as their mode of action. Moreover, the continuous spectrophotometric assay gave similar I_{50} values to those obtained with the $^{14}CO_2$ assay. The spectrophotometric assay can be adapted to a high capacity screen for

inhibitors of *p*-hydroxyphenylpyruvate dioxygenase by adapting it to a microtiter plate assay combined with a plate reader that would read at or near 308 nm. Furthermore, any colorimetric or fluorescent assay for homogentisate or *p*-hydroxyphenylpyruvate would also be able to be readily adapted into a high capacity screen for inhibitors of this enzyme. The isolated overexpressed enzyme has sufficient activity to be used directly in a spectrophotometric assay or it can be further purified for enhanced assay sensitivity.

EXAMPLE 5

Re-construction of the Full-length *p*-Hydroxyphenylpyruvate Dioxygenase Gene for Production of Active, Stable Enzyme in Bacteria

The plasmid pT7BlueR+PDO2, described in Example 2 and containing the full-length *p*-hydroxyphenylpyruvate dioxygenase gene, proved to have incorrect sequence at the EcoR1 site. This was re-sequenced so that an oligonucleotide could be designed to replace the EcoRI site with an NdeI site using conventional loop-out mutagenesis. The oligonucleotide was designed so that this procedure also introduced an ATG initiation codon at the 5'- end of the *p*-hydroxyphenylpyruvate dioxygenase gene followed by the full-length *p*-hydroxyphenylpyruvate dioxygenase sequence. After mutagenesis, the clone was amplified in *E. coli* and the plasmid was purified. The resulting full-length gene, "PDO-B", was then digested with the enzymes using NdeI and NheI, and the ~820 bp fragment used to replace the NdeI - Nhe I segment of the truncated *p*-hydroxyphenylpyruvate dioxygenase gene, "PDO-A," in pE24CP1 (Example 1). The resulting plasmid, pE24PDO-B can be expressed in bacteria to produce the full-length *Arabidopsis* *p*-hydroxyphenylpyruvate dioxygenase enzyme as determined by enzyme activity and N-terminal sequence analysis.

EXAMPLE 6

Enhanced Stability of Full Length Construct Over the Truncated Construct

Two different constructs for *Arabidopsis thaliana* *p*-hydroxyphenylpyruvate dioxygenase, one containing the full-length sequence, PDO-B as described in Example 5 and produced from plasmid pE24PDO-B, and one containing the truncated sequence lacking the putative chloroplast leader sequence, PDO-A as produced from plasmid pE24CP1, were both purified to the same extent using a Pharmacia phenyl Sepharose hydrophobic interaction column followed by gel filtration chromatography on Pharmacia Sephacryl 300. The two proteins were diluted to 1 mg/mL in 20 mM bis tris-propane buffer, pH 7.2 containing 5 mM ascorbate, 1 mM reduced glutathione and 0.1 mM ferrous ammonium sulfate and stored in a refrigerator at 4 °C for up to 10 days. Aliquots were removed at various times and assayed for activity using the tautomerase

coupled spectrophotometric assay. Under these conditions the half-life for the activity of the full length enzyme was 4 days, whereas the truncated enzyme preparation had a half-life of 9 to 10 hours. In addition, the activity of the full length enzyme could be restored by incubation with iron and reducing agent, reduced glutathione or ascorbate, or by dialysis against buffer containing iron and reducing agent. In contrast, the activity of the truncated enzyme could not be restored by incubation with or dialysis against buffer containing iron and reducing agent. The full-length enzyme was also more stable in the spectrophotometric assay showing a 2 to 3 times longer useful linear region than the truncated enzyme. Both enzyme preparations showed similar I_{50} values with the herbicidally active inhibitors.

These results clearly show that the full-length PDO-B construct has decided advantages over the truncated enzyme due to the enhanced stability under storage conditions, in the spectrophotometric assay and in the reversible reconstitution of activity in the presence of iron and reducing agent. While both enzyme constructs can be used for screening of inhibitors, the PDO-B enzyme is preferred for this application and is far superior for mechanistic and structural studies.

EXAMPLE 7

Cloning of the Maize *p*-Hydroxyphenylpyruvate Dioxygenase Gene

Approximately 600,000 plaques of a Stratagene maize Uni-Zap cDNA library (from young plants) were screened by filter hybridization under moderate stringency using a heterologous probe. The probe was prepared by PCR and was a 916 bp fragment of DNA having the sequence defined by the region extending from position 263 to 1178 of SEQ ID NO:14. Twenty-four positive phage clones were identified in the primary screen, and eleven phage clones were recovered from a secondary screen. Seven positive clones were submitted for sequencing, and four showed significant conservation sequence at the amino acid level when compared with the *Arabidopsis thaliana* *p*-hydroxyphenylpyruvate dioxygenase protein. The longest of the four contained an insert of 988 bp and showed 70% identity and 78% similarity with the *Arabidopsis* protein, but was lacking approximately 550 bp corresponding to the amino terminal end of the protein.

Attempts to obtain a full-length cDNA of the maize *p*-hydroxyphenylpyruvate dioxygenase gene were unsuccessful, possibly because the secondary structure of the RNA inhibited efficient reverse transcription of this transcript. Two additional cDNA libraries were screened and clones long enough to contain a full-length cDNA were sequenced. All of these clones were shown to be chimeras. Therefore a genomic library was screened to obtain the 5' one-third of

the gene. Approximately 1 million clones from a Clontech *Zea mays* (var. B73) library in the phage vector EMBL3 (whole seedlings, 2 leaf stage) were screened using a 415 bp EcoRI-BssHII fragment containing the 5' end of the truncated corn *p*-hydroxyphenylpyruvate dioxygenase cDNA (clone H1011C). Eight positive primary phage clones were plated and screened, and four secondary clones were picked. DNA was prepared from each using the Qiagen Lambda midi-kit. Restriction digests with SalI or EcoRI indicated that two clones were the same. DNA samples from the remaining 3 clones (11.1.3, 13.1.1, and 21.2.1) were digested with SalI, EcoRI, or SalI and EcoRI, prepared for Southern analysis, and probed with the full length *Arabidopsis p*-hydroxyphenylpyruvate dioxygenase gene. Two of the clones (11.1.3 and 13.1.1) showed sequence conservation, and these homologous fragments were subcloned and sequenced. Both clones appeared to contain the full-length gene and each contained one intron near the 3' end of the gene. However, there were differences between the sequences of the two clones indicating that they may be two different genes or one may be a pseudogene. The sequence of clone 11.1.3 matched the cDNA sequence, and this clone was used to construct a full length *p*-hydroxyphenylpyruvate dioxygenase coding region.

The gene was contained on two adjacent fragments, a 3.5 kb EcoRI - SalI fragment and a 2 kb SalI fragment. Both were subcloned into pBluescript SKII+ resulting in the plasmids pES1113 and pSal1113. pES1113 was digested with SpeI to release approximately 2.7 kb of upstream sequence and then religated, resulting in a plasmid with an insert of 747 base pairs (pSPE1). pSPE1 was digested with SalI to linearize the plasmid and ligated with the 2 kb SalI fragment from pSal1113, which had been released by digestion with SalI and gel purified. Orientation was confirmed by digestion with SpeI and Bpu1102I and the correct plasmid was named p1113. In order to remove the intron contained in the 3' end of the genomic clone, the plasmid was digested with Bpu1102I and XhoI and the 3.9 kb fragment containing the vector and 5' part of the gene was gel purified. The corresponding 882 bp Bpu1102I-XhoI fragment from pH1011c (cDNA)was gel purified and ligated with this 3.9 kb fragment resulting in the clone pMPDO (ATCC 209120), which contains a 1782 bp insert. There are 260 base pairs upstream of the putative ATG and 189 base pairs downstream of the stop codon. The full-length sequence was confirmed by sequencing across the insert. The nucleic acid sequence and the deduced protein sequence for corn *p*-hydroxyphenylpyruvate dioxygenase are presented in SEQ ID NOS:10 and 11, respectively. The sequences for *p*-hydroxyphenylpyruvate dioxygenases obtained from corn and *Arabidopsis* were compared using the "Gap" program of GCG

(Program Manual for the Wisconsin Package, Version 9.0-OpenVMS. December 1996, Genetics Computer Group. 575 Science Drive. Madison. WI, USA 53711). The results of these comparisons indicated that these functions are approximately 67% identical at the nucleotide level, and they possess 69% similarity and 62% identity at the amino acid level. The predicted amino acid sequence of corn *p*-hydroxyphenylpyruvate dioxygenase is compared with that from *Arabidopsis* and other eukaryotes in Figure 3.

EXAMPLE 8

Composition of a cDNA Library; Isolation and Sequencing of cDNA Clones

A cDNA library representing mRNAs from developing seeds of *Vernonia galamenensis* that had just begun production of vernolic acid was prepared. The library was prepared in a Uni-ZAP™ XR vector according to the manufacturer's protocol (Stratagene Cloning Systems, La Jolla, CA). Conversion of the Uni-ZAP™ XR library into a plasmid library was accomplished according to the protocol provided by Stratagene. Upon conversion, cDNA inserts were contained in the plasmid vector pBluescript. cDNA inserts from randomly picked bacterial colonies containing recombinant pBluescript plasmids were amplified via polymerase chain reaction using primers specific for vector sequences flanking the inserted cDNA sequences. Amplified insert DNAs were sequenced in dye-primer sequencing reactions to generate partial cDNA sequences (expressed sequence tags or "ESTs"; see Adams, M. D. et al., (1991) *Science 252*:1651). The resulting ESTs were analyzed using a Perkin Elmer Model 377 fluorescent sequencer.

EXAMPLE 9

Identification and Characterization of cDNA Clones

ESTs encoding *Vernonia galamenensis* enzymes were identified by conducting BLAST (Basic Local Alignment Search Tool; Altschul, S. F. et al., (1993) *J. Mol. Biol.* 215:403-410; see also www.ncbi.nlm.nih.gov/BLAST/) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). The cDNA sequences obtained in Example 9 were analyzed for similarity to all publicly available DNA sequences contained in the "nr" database using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequences were translated in all reading frames and compared for similarity to all publicly available protein sequences contained in the "nr" database using the BLASTX algorithm (Gish, W. and States, D. J.

(1993) *Nature Genetics* 3:266-272) provided by the NCBI. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

The BLASTX search using clone vs1.pk0015.b2 revealed similarity of the protein encoded by the cDNA to a number of *p*-hydroxyphenylpyruvate dioxygenases from sources other that plants. The three most similar *p*-hydroxyphenylpyruvate dioxygenase proteins were a streptomycete *p*-hydroxyphenylpyruvate dioxygenase (GenBank Accession No. U11864; pLog = 8.34), a rat *p*-hydroxyphenylpyruvate dioxygenase (GenBank Accession No. M18405; pLog = 7.66), and a human *p*-hydroxyphenylpyruvate dioxygenase (GenBank Accession No. U29895; pLog = 7.60). SEQ ID NO:16 shows the nucleotide sequence of a portion of the *Vernonia galamenensis* cDNA in clone vs1.pk0015.b2. Sequence alignments and BLAST scores and probabilities indicate that the instant nucleic acid fragment encodes a portion of *Vernonia galamenensis* *p*-hydroxyphenylpyruvate dioxygenase.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: E. I. DUPONT DE NEMOURS AND COMPANY
(B) STREET: 1007 MARKET STREET
(C) CITY: WILMINGTON
(D) STATE: DELAWARE
(E) COUNTRY: U.S.A.
(F) POSTAL CODE (ZIP): 19898
(G) TELEPHONE: 302-892-8112
(H) TELEFAX: 302-773-0164
(I) TELEX: 6717325

(ii) TITLE OF INVENTION: PLANT GENE FOR *p*-HYDROXY-PHENYLPYRUVATE DIOXYGENASE

(iii) NUMBER OF SEQUENCES: 16

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: DISKETTE, 3.50 INCH
(B) COMPUTER: IBM PC COMPATIBLE
(C) OPERATING SYSTEM: MICROSOFT WORD FOR WINDOWS 95
(D) SOFTWARE: MICROSOFT WORD VERSION 7.0A

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/021,364
(B) FILING DATE: JUNE 27, 1996

(vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: FLOYD, LINDA AXAMETHY
(B) REGISTRATION NUMBER: 33,692
(C) REFERENCE/DOCKET NUMBER: BA-9120

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 233 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
CAAGAAACGN GTCGNCGACG TGCTCAGCGA TGATCAGATC AAGGAGTGTG AGGAATTAGG  60

GATTCTTNTA GACAGAGATG ATCAAGGGAC GTTNCTTCAA ATCTNCACAA AACCACTAGG 120

TGACAGGCCG ACGNTATTTA TAGAGATAAT CCAGAGNGTA GGATGCATGA TGAAAGATGT 180

GGAAGGGANG GCTTACCAGA GTGGAGNATN TNGTGGTTTT GGCAAAGGCA ATT        233
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1448 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 9..1343

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
TGAAATCA ATG GGC CAC CAA AAC GCC GCC GTT TCA GAG AAT CAA AAC CAT      50
         Met Gly His Gln Asn Ala Ala Val Ser Glu Asn Gln Asn His
           1               5                  10

GAT GAC GGC GCT GCG TCG TCG CCG GGA TTC AAG CTC GTC GGA TTT TCC       98
Asp Asp Gly Ala Ala Ser Ser Pro Gly Phe Lys Leu Val Gly Phe Ser
 15                  20                  25                  30

AAG TTC GTA AGA AAG AAT CCA AAG TCT GAT AAA TTC AAG GTT AAG CGC      146
Lys Phe Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val Lys Arg
                 35                  40                  45

TTC CAT CAC ATC GAG TTC TGG TGC GGG GAC GCA ACC AAC GTC GCT CGT      194
Phe His His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val Ala Arg
             50                  55                  60

CGC TTC TCC TGG GGT CTG GGG ATG AGA TTC TCC GCC AAA TCC GAT CTT      242
Arg Phe Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser Asp Leu
         65                  70                  75

TCC ACC GGA AAC ATG GTT CAC GCC TCT TAC CTA CTC ACC TCC GGT GAA      290
Ser Thr Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser Gly Glu
     80                  85                  90

CTC CGA TTC CTT TTC ACT GCT CCT TAC TCT CCG TCT CTC TCC GGC GGA      338
Leu Arg Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser Gly Gly
 95                 100                 105                 110
```

```
GAG ATT AAA CCG ACA ACC ACA GGT TCT ATC CCA AGT TTC GAT CAC GGG     386
Glu Ile Lys Pro Thr Thr Thr Gly Ser Ile Pro Ser Phe Asp His Gly
                115                 120                 125

TCT TGT CGG TCC TTC TTC TCT TCA CAT GGT CTC GGT GTT AGA CCC GTT     434
Ser Cys Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg Pro Val
            130                 135                 140

GCG ATT GAA GTA GAA GAC GCG GAG TCA GCT TTC TCC ATC AGT GTA GCT     482
Ala Ile Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser Val Ala
        145                 150                 155

AAT GGC GCT ATT CCT TCG TCG CCT CCT ATC GTC CTC AAT GAA GCA GTT     530
Asn Gly Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu Ala Val
    160                 165                 170

ACG ATC GCT GAG GTT AAA CTA TAC GGC GAT GTT GTT CTC CGA TAT GTT     578
Thr Ile Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg Tyr Val
175                 180                 185                 190

AGT TAC AAA GCA GAA GAT ACC GAA AAA TCC GAA TTC TTG CCA GGG TTC     626
Ser Tyr Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro Gly Phe
                195                 200                 205

GAG CGT GTA GAG GAT GCG TCG TCG TTC CCA TTG GAT TAT GGT ATC CGG     674
Glu Arg Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly Ile Arg
            210                 215                 220

CGG CTT GAC CAC GCC GTG GGA AAC GTT CCT GAG CTT GGT CCG GCT TTA     722
Arg Leu Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro Ala Leu
        225                 230                 235

ACT TAT GTA GCG GGG TTC ACT GGT TTT CAC CAA TTC GCA GAG TTC ACA     770
Thr Tyr Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu Phe Thr
    240                 245                 250

GCA GAC GAC GTT GGA ACC GCC GAG AGC GGT TTA AAT TCA GCG GTC CTG     818
Ala Asp Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala Val Leu
255                 260                 265                 270

GCT AGC AAT GAT GAA ATG GTT CTT CTA CCG ATT AAC GAG CCA GTG CAC     866
Ala Ser Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro Val His
                275                 280                 285

GGA ACA AAG AGG AAG AGT CAG ATT CAG ACG TAT TTG GAA CAT AAC GAA     914
Gly Thr Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His Asn Glu
            290                 295                 300

GGC GCA GGG CTA CAA CAT CTG GCT CTG ATG AGT GAA GAC ATA TTC AGG     962
Gly Ala Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile Phe Arg
        305                 310                 315

ACC CTG AGA GAG ATG AGG AAG AGG AGC AGT ATT GGA GGA TTC GAC TTC    1010
Thr Leu Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe Asp Phe
    320                 325                 330

ATG CCT TCT CCT CCG CCT ACT TAC TAC CAG AAT CTC AAG AAA CGG GTC    1058
Met Pro Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys Arg Val
335                 340                 345                 350

GGC GAC GTG CTC AGC GAT GAT CAG ATC AAG GAG TGT GAG GAA TTA GGG    1106
Gly Asp Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu Leu Gly
                355                 360                 365
```

```
ATT CTT GTA GAC AGA GAT GAT CAA GGG ACG TTG CTT CAA ATC TTC ACA     1154
Ile Leu Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile Phe Thr
            370                 375                 380

AAA CCA CTA GGT GAC AGG CCG ACG ATA TTT ATA GAG ATA ATC CAG AGA     1202
Lys Pro Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu Ile Ile Gln Arg
        385                 390                 395

GTA GGA TGC ATG ATG AAA GAT GAG GAA GGG AAG GCT TAC CAG AGT GGA     1250
Val Gly Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln Ser Gly
    400                 405                 410

GGA TGT GGT GGT TTT GCC AAA GGC AAT TTC TCT GAG CTC TTC AAG TCC     1298
Gly Cys Gly Gly Phe Ala Lys Gly Asn Phe Ser Glu Leu Phe Lys Ser
415                 420                 425                 430

ATT GAA GAA TAC GAA AAG ACT CTT GAA GCC AAA CAG TTA GTG GGA         1343
Ile Glu Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val Gly
                435                 440                 445

TGAACAAGAA GAAGAACCAA CTAAAGGATT GTGTAATTAA TGTAAAACTG TTTTATCTTA 1403

TCAAAACAAT GTATACAACA TCTCATTTAA AAACGAGATC AATCC                 1448
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 445 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Met Gly His Gln Asn Ala Ala Val Ser Glu Asn Gln Asn His Asp Asp
  1               5                  10                  15

Gly Ala Ala Ser Ser Pro Gly Phe Lys Leu Val Gly Phe Ser Lys Phe
             20                  25                  30

Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val Lys Arg Phe His
         35                  40                  45

His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val Ala Arg Arg Phe
     50                  55                  60

Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser Asp Leu Ser Thr
 65                  70                  75                  80

Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser Gly Glu Leu Arg
                 85                  90                  95

Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser Gly Gly Glu Ile
            100                 105                 110

Lys Pro Thr Thr Thr Gly Ser Ile Pro Ser Phe Asp His Gly Ser Cys
        115                 120                 125

Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg Pro Val Ala Ile
    130                 135                 140

Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser Val Ala Asn Gly
145                 150                 155                 160
```

```
Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu Ala Val Thr Ile
                165                 170                 175

Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg Tyr Val Ser Tyr
            180                 185                 190

Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro Gly Phe Glu Arg
        195                 200                 205

Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly Ile Arg Arg Leu
    210                 215                 220

Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro Ala Leu Thr Tyr
225                 230                 235                     240

Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu Phe Thr Ala Asp
                245                 250                 255

Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala Val Leu Ala Ser
            260                 265                 270

Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro Val His Gly Thr
        275                 280                 285

Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His Asn Glu Gly Ala
    290                 295                 300

Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile Phe Arg Thr Leu
305                 310                 315                     320

Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe Asp Phe Met Pro
                325                 330                 335

Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys Arg Val Gly Asp
            340                 345                 350

Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu Leu Gly Ile Leu
        355                 360                 365

Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile Phe Thr Lys Pro
    370                 375                 380

Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu Ile Ile Gln Arg Val Gly
385                 390                 395                     400

Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln Ser Gly Gly Cys
                405                 410                 415

Gly Gly Phe Ala Lys Gly Asn Phe Ser Glu Leu Phe Lys Ser Ile Glu
            420                 425                 430

Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val Gly
        435                 440                 445
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 53 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

TATGTCCAAG TTCGTAAGAA AGAATCCAAA GTCTGATAAA TTCAAGGTTA AGC 53

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GCTTAACCTT GAATTTATCA GACTTTGGAT TCTTTCTTAC GAACTTGGAC A 51

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 392 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Thr Ser Tyr Ser Asp Lys Gly Glu Lys Pro Glu Arg Gly Arg Phe Leu
1 5 10 15

His Phe His Ser Val Thr Phe Trp Val Gly Asn Ala Lys Gln Ala Ala
20 25 30

Ser Tyr Tyr Cys Ser Lys Ile Gly Phe Glu Pro Leu Ala Tyr Lys Gly
35 40 45

Leu Glu Thr Gly Ser Arg Glu Val Val Ser His Val Val Lys Gln Asp
50 55 60

Lys Ile Val Phe Val Phe Ser Ser Ala Leu Asn Pro Trp Asn Lys Glu
65 70 75 80

Met Gly Asp His Leu Val Lys His Gly Asp Gly Val Lys Asp Ile Ala
85 90 95

Phe Glu Val Glu Asp Cys Asp Tyr Ile Val Gln Lys Ala Arg Glu Arg
100 105 110

Gly Ala Ile Ile Val Arg Glu Glu Val Cys Cys Ala Ala Asp Val Arg
115 120 125

Gly His His Thr Pro Leu Asp Arg Ala Arg Gln Val Trp Glu Gly Thr
130 135 140

Leu Val Glu Lys Met Thr Phe Cys Leu Asp Ser Arg Pro Gln Pro Ser
145 150 155 160

Gln Thr Leu Leu His Arg Leu Leu Leu Ser Lys Leu Pro Lys Cys Gly
165 170 175

Leu Glu Ile Ile Asp His Ile Val Gly Asn Gln Pro Asp Gln Glu Met
180 185 190

```
Glu Ser Ala Ser Gln Trp Tyr Met Arg Asn Leu Gln Phe His Arg Phe
        195                 200                 205

Trp Ser Val Asp Asp Thr Gln Ile His Thr Glu Tyr Ser Ala Leu Arg
    210                 215                 220

Ser Val Val Met Ala Asn Tyr Glu Glu Ser Ile Lys Met Pro Ile Asn
225                 230                 235                 240

Glu Pro Ala Pro Gly Lys Lys Lys Ser Gln Ile Gln Glu Tyr Val Asp
                245                 250                 255

Tyr Asn Gly Gly Ala Gly Val Gln His Ile Ala Leu Lys Thr Glu Asp
            260                 265                 270

Ile Ile Thr Ala Ile Arg Ser Leu Arg Glu Arg Gly Val Glu Phe Leu
        275                 280                 285

Ala Val Pro Phe Thr Tyr Tyr Lys Gln Leu Gln Glu Lys Leu Lys Ser
    290                 295                 300

Ala Lys Ile Arg Val Lys Glu Ser Ile Asp Val Leu Glu Glu Leu Lys
305                 310                 315                 320

Ile Leu Val Asp Tyr Asp Glu Lys Gly Tyr Leu Leu Gln Ile Phe Thr
                325                 330                 335

Lys Pro Met Gln Asp Arg Pro Thr Val Phe Leu Glu Val Ile Gln Arg
            340                 345                 350

Asn Asn His Gln Gly Phe Gly Ala Gly Asn Phe Asn Ser Leu Phe Lys
        355                 360                 365

Ala Phe Glu Glu Glu Gln Glu Leu Arg Gly Asn Leu Thr Asp Thr Asp
    370                 375                 380

Pro Asn Gly Val Pro Phe Arg Leu
385                 390
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 392 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Thr Ser Tyr Ser Asp Lys Gly Glu Lys Pro Glu Arg Gly Arg Phe Leu
1               5                   10                  15

His Phe His Ser Val Thr Phe Trp Val Gly Asn Ala Lys Gln Ala Ala
            20                  25                  30

Ser Tyr Tyr Cys Ser Lys Ile Gly Phe Glu Pro Leu Ala Tyr Lys Gly
        35                  40                  45

Leu Glu Thr Gly Ser Arg Glu Val Val Ser His Val Val Lys Gln Asp
    50                  55                  60

Lys Ile Val Phe Val Phe Ser Ser Ala Leu Asn Pro Trp Asn Lys Glu
65                  70                  75                  80
```

Met Gly Asp His Leu Val Lys His Gly Asp Gly Val Lys Asp Ile Ala
85 90 95

Phe Glu Val Glu Asp Cys Asp Tyr Ile Val Gln Lys Ala Arg Glu Arg
100 105 110

Gly Ala Ile Ile Val Arg Glu Glu Val Cys Cys Ala Ala Asp Val Arg
115 120 125

Gly His His Thr Pro Leu Asp Arg Ala Arg Gln Val Trp Glu Gly Thr
130 135 140

Leu Val Glu Lys Met Thr Phe Cys Leu Asp Ser Arg Pro Gln Pro Ser
145 150 155 160

Gln Thr Leu Leu His Arg Leu Leu Leu Ser Lys Leu Pro Lys Cys Gly
165 170 175

Leu Glu Ile Ile Asp His Ile Val Gly Asn Gln Pro Asp Gln Glu Met
180 185 190

Glu Ser Ala Ser Gln Trp Tyr Met Arg Asn Leu Gln Phe His Arg Phe
195 200 205

Trp Ser Val Asp Asp Thr Gln Ile His Thr Glu Tyr Ser Ala Leu Arg
210 215 220

Ser Val Val Met Ala Asn Tyr Glu Glu Ser Ile Lys Met Pro Ile Asn
225 230 235 240

Glu Pro Ala Pro Gly Lys Lys Lys Ser Gln Ile Gln Glu Tyr Val Asp
245 250 255

Tyr Asn Gly Gly Ala Gly Val Gln His Ile Ala Leu Lys Thr Glu Asp
260 265 270

Ile Ile Thr Ala Ile Arg Ser Leu Arg Glu Arg Gly Val Glu Phe Leu
275 280 285

Ala Val Pro Phe Thr Tyr Tyr Lys Gln Leu Gln Glu Lys Leu Lys Ser
290 295 300

Ala Lys Ile Arg Val Lys Glu Ser Ile Asp Val Leu Glu Glu Leu Lys
305 310 315 320

Ile Leu Val Asp Tyr Asp Glu Lys Gly Tyr Leu Leu Gln Ile Phe Thr
325 330 335

Lys Pro Met Gln Asp Arg Pro Thr Val Phe Leu Glu Val Ile Gln Arg
340 345 350

Asn Asn His Gln Gly Phe Gly Ala Gly Asn Phe Asn Ser Leu Phe Lys
355 360 365

Ala Phe Glu Glu Glu Gln Glu Leu Arg Gly Asn Leu Thr Asp Thr Asp
370 375 380

Pro Asn Gly Val Pro Phe Arg Leu
385 390

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 392 amino acids
(B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Thr Thr Tyr Asn Asn Lys Gly Pro Lys Pro Glu Arg Gly Arg Phe Leu
1               5                   10                  15

His Phe His Ser Val Thr Phe Trp Val Gly Asn Ala Lys Gln Ala Ala
            20                  25                  30

Ser Phe Tyr Cys Asn Lys Met Gly Phe Glu Pro Leu Ala Tyr Arg Gly
        35                  40                  45

Leu Glu Thr Gly Ser Arg Glu Val Val Ser His Val Ile Lys Arg Gly
    50                  55                  60

Lys Ile Val Phe Val Leu Cys Ser Ala Leu Asn Pro Trp Asn Lys Glu
65                  70                  75                  80

Met Gly Asp His Leu Val Lys His Gly Asp Gly Val Lys Asp Ile Ala
                85                  90                  95

Phe Glu Val Glu Asp Cys Asp His Ile Val Gln Lys Ala Arg Glu Arg
            100                 105                 110

Gly Ala Lys Ile Val Arg Glu Pro Trp Val Glu Gln Asp Lys Phe Gly
        115                 120                 125

Lys Val Lys Phe Ala Val Leu Gln Thr Tyr Gly Asp Thr Thr His Thr
    130                 135                 140

Leu Val Glu Lys Ile Asn Tyr Thr Gly Arg Phe Leu Pro Gly Phe Glu
145                 150                 155                 160

Ala Pro Thr Tyr Lys Asp Thr Leu Leu Pro Lys Leu Pro Arg Cys Asn
                165                 170                 175

Leu Glu Ile Ile Asp His Ile Val Gly Asn Gln Pro Asp Gln Glu Met
            180                 185                 190

Gln Ser Ala Ser Glu Trp Tyr Leu Lys Asn Leu Gln Phe His Arg Phe
        195                 200                 205

Trp Ser Val Asp Asp Thr Gln Val His Thr Glu Tyr Ser Ser Leu Arg
    210                 215                 220

Ser Ile Val Val Thr Asn Tyr Glu Glu Ser Ile Lys Met Pro Ile Asn
225                 230                 235                 240

Glu Pro Ala Pro Gly Arg Lys Lys Ser Gln Ile Gln Glu Tyr Val Asp
                245                 250                 255

Tyr Asn Gly Gly Ala Gly Val Gln His Ile Ala Leu Lys Thr Glu Asp
            260                 265                 270

Ile Ile Thr Ala Ile Arg His Leu Arg Glu Arg Gly Thr Glu Phe Leu
        275                 280                 285

Ala Ala Pro Ser Ser Tyr Tyr Lys Leu Leu Arg Glu Asn Leu Lys Ser
    290                 295                 300

Ala Lys Ile Gln Val Lys Glu Ser Met Asp Val Leu Glu Glu Leu His
305                 310                 315                 320
```

Ile Leu Val Asp Tyr Asp Glu Lys Gly Tyr Leu Leu Gln Ile Phe Thr
325 330 335

Lys Pro Met Gln Asp Arg Pro Thr Leu Phe Leu Glu Val Ile Gln Arg
340 345 350

His Asn His Gln Gly Phe Gly Ala Gly Asn Phe Asn Ser Leu Phe Lys
355 360 365

Ala Phe Glu Glu Glu Gln Ala Leu Arg Gly Asn Leu Thr Asp Leu Glu
370 375 380

Pro Asn Gly Val Arg Ser Gly Met
385 390

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 376 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Tyr Trp Asp Lys Gly Pro Lys Pro Glu Arg Gly Arg Phe Leu His Phe
1 5 10 15

His Ser Val Thr Phe Trp Val Gly Asn Ala Lys Gln Ala Ala Ser Phe
20 25 30

Tyr Cys Asn Lys Met Gly Phe Glu Pro Leu Ala Tyr Lys Gly Leu Glu
35 40 45

Thr Gly Ser Arg Glu Val Val Ser His Val Ile Lys Gln Gly Lys Ile
50 55 60

Val Phe Val Leu Cys Ser Ala Leu Asn Pro Trp Asn Lys Glu Met Gly
65 70 75 80

Asp His Leu Val Lys His Gly Asp Gly Val Lys Asp Ile Ala Phe Glu
85 90 95

Val Glu Asp Cys Glu His Ile Val Gln Lys Ala Arg Glu Arg Gly Ala
100 105 110

Lys Ile Val Arg Glu Pro Trp Val Glu Glu Asp Lys Phe Gly Lys Val
115 120 125

Lys Phe Ala Val Leu Gln Thr Tyr Gly Asp Thr Thr His Thr Leu Val
130 135 140

Glu Lys Ile Asn Tyr Thr Gly Arg Phe Leu Pro Gly Phe Glu Ala Pro
145 150 155 160

Thr Tyr Lys Asp Thr Leu Leu Pro Lys Leu Pro Ser Cys Asn Leu Glu
165 170 175

Ile Ile Asp His Ile Val Gly Asn Gln Pro Asp Gln Glu Met Glu Ser
180 185 190

Ala Ser Glu Trp Tyr Leu Lys Asn Leu Gln Phe His Arg Phe Trp Ser
195 200 205

Val Asp Asp Thr Gln Val His Thr Glu Tyr Ser Ser Leu Arg Ser Ile
210 215 220

Val Val Ala Asn Tyr Glu Glu Ser Ile Lys Met Pro Ile Asn Glu Pro
225 230 235 240

Ala Pro Gly Arg Lys Lys Ser Gln Ile Gln Glu Tyr Val Asp Tyr Asn
245 250 255

Gly Gly Ala Gly Val Gln His Ile Ala Leu Arg Thr Glu Asp Ile Ile
260 265 270

Thr Thr Ile Arg His Leu Arg Glu Arg Gly Met Glu Phe Leu Ala Val
275 280 285

Pro Ser Ser Tyr Tyr Arg Leu Leu Arg Glu Asn Leu Lys Thr Ser Lys
290 295 300

Ile Gln Val Lys Glu Asn Met Asp Val Leu Glu Glu Leu Lys Ile Leu
305 310 315 320

Val Asp Tyr Asp Glu Lys Gly Tyr Leu Leu Gln Ile Phe Thr Lys Pro
325 330 335

Met Gln Asp Arg Pro Thr Leu Phe Leu Glu Val Ile Gln Arg His Asn
340 345 350

His Gln Gly Phe Gly Ala Gly Asn Phe Asn Ser Leu Phe Lys Ala Phe
355 360 365

Glu Glu Glu Gln Ala Leu Arg Gly
370 375

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1766 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Zea mays

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 261..1595

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACTAGTTGTG AGAGCCTTCT GCGTTGGCAA TTGGCAGTAC AAGACAAATC ACATCCGCAA 60

CCGCAACCAC AGAATCGTCC GTCCACGTGG CCCCCATCAC TTCCCTTTAT TTACCAGTCG 120

TCCCCCATCC CCAGGGCCAC CCACCAACAA GTGCAGTCAC CCGAGCCGCA AACTGCAGCT 180

CTGCAAGCTA CAGAGGCCAC CACGAGTCCA CGACGCCACG CCCTCCGAGA GAAAGAGAAA 240

```
GAGAAAACCA AAGCACGATA ATG CCC CCG ACC CCC ACA GCC GCC GCA GCC     290
                      Met Pro Pro Thr Pro Thr Ala Ala Ala Ala
                        1               5                  10

GGC GCC GCC GTG GCG GCG GCA TCA GCA GCG GAG CAA GCG GCG TTC CGC   338
Gly Ala Ala Val Ala Ala Ala Ser Ala Ala Glu Gln Ala Ala Phe Arg
                 15                  20                  25

CTC GTG GGC CAC CGC AAC TTC GTC CGC TTC AAC CCG CGC TCC GAC CGC   386
Leu Val Gly His Arg Asn Phe Val Arg Phe Asn Pro Arg Ser Asp Arg
             30                  35                  40

TTC CAC ACG CTC GCG TTC CAC CAC GTG GAG CTC TGG TGC GCC GAC GCG   434
Phe His Thr Leu Ala Phe His His Val Glu Leu Trp Cys Ala Asp Ala
         45                  50                  55

GCC TCC GCC GCG GGC CGC TTC TCC TTC GGC CTG GGC GCG CCG CTC GCC   482
Ala Ser Ala Ala Gly Arg Phe Ser Phe Gly Leu Gly Ala Pro Leu Ala
     60                  65                  70

GCA CGC TCC GAC CTC TCC ACG GGC AAC TCC GCG CAC GCG TCC CTG CTG   530
Ala Arg Ser Asp Leu Ser Thr Gly Asn Ser Ala His Ala Ser Leu Leu
 75                  80                  85                  90

CTC CGC TCC GGC TCC CTC TCC TTC CTC TTC ACG GCG CCC TAC GCG CAC   578
Leu Arg Ser Gly Ser Leu Ser Phe Leu Phe Thr Ala Pro Tyr Ala His
                 95                 100                 105

GGC GCC GAC GCT GCC ACC GCC GCG CTG CCC TCC TTC TCC GCC GCC GCC   626
Gly Ala Asp Ala Ala Thr Ala Ala Leu Pro Ser Phe Ser Ala Ala Ala
            110                 115                 120

GCG CGG CGC TTC GCA GCC GAC CAC GGC CTC GCG GTG CGC GCC GTC GCG   674
Ala Arg Arg Phe Ala Ala Asp His Gly Leu Ala Val Arg Ala Val Ala
        125                 130                 135

CTC CGC GTC GCC GAC GCC GAG GAC GCC TTC CGC GCC AGC GTC GCG GCC   722
Leu Arg Val Ala Asp Ala Glu Asp Ala Phe Arg Ala Ser Val Ala Ala
    140                 145                 150

GGG GCG CGC CCG GCG TTC GGC CCC GTC GAC CTC GGC CGC GGC TTC CGC   770
Gly Ala Arg Pro Ala Phe Gly Pro Val Asp Leu Gly Arg Gly Phe Arg
155                 160                 165                 170

CTC GCC GAG GTC GAG CTC TAC GGC GAC GTC GTG CTC CGG TAC GTG AGC   818
Leu Ala Glu Val Glu Leu Tyr Gly Asp Val Val Leu Arg Tyr Val Ser
                175                 180                 185

TAC CCG GAC GGC GCC GCG GGC GAG CCC TTC CTG CCG GGG TTC GAG GGC   866
Tyr Pro Asp Gly Ala Ala Gly Glu Pro Phe Leu Pro Gly Phe Glu Gly
            190                 195                 200

GTG GCC AGC CCC GGG GCG GCC GAC TAC GGG CTG AGC AGG TTC GAC CAC   914
Val Ala Ser Pro Gly Ala Ala Asp Tyr Gly Leu Ser Arg Phe Asp His
        205                 210                 215

ATC GTC GGC AAC GTG CCG GAG CTG GCG CCC GCC GCC GCC TAC TTC GCC   962
Ile Val Gly Asn Val Pro Glu Leu Ala Pro Ala Ala Ala Tyr Phe Ala
    220                 225                 230

GGC TTC ACG GGG TTC CAC GAG TTC GCC GAG TTC ACG ACG GAG GAC GTG  1010
Gly Phe Thr Gly Phe His Glu Phe Ala Glu Phe Thr Thr Glu Asp Val
235                 240                 245                 250
```

GGC ACC GCG GAG AGC GGC CTC AAC TCC ATG GTG CTC GCC AAC AAC TCG 1058
Gly Thr Ala Glu Ser Gly Leu Asn Ser Met Val Leu Ala Asn Asn Ser
255 260 265

GAG AAC GTG CTG CTC CCG CTC AAC GAG CCG GTG CAC GGC ACC AAG CGC 1106
Glu Asn Val Leu Leu Pro Leu Asn Glu Pro Val His Gly Thr Lys Arg
270 275 280

CGC AGC CAG ATA CAA ACG TTC CTG GAC CAC CAC GGC GGC CCC GGC GTG 1154
Arg Ser Gln Ile Gln Thr Phe Leu Asp His His Gly Gly Pro Gly Val
285 290 295

CAG CAC ATG GCG CTG GCC AGC GAC GAC GTG CTC AGG ACG CTG AGG GAG 1202
Gln His Met Ala Leu Ala Ser Asp Asp Val Leu Arg Thr Leu Arg Glu
300 305 310

ATG CAG GCG CGC TCG GCC ATG GGC GGC TTC GAG TTC ATG GCG CCT CCC 1250
Met Gln Ala Arg Ser Ala Met Gly Gly Phe Glu Phe Met Ala Pro Pro
315 320 325 330

ACA TCC GAC TAC TAT GAC GGC GTG AGG CGG CGC GCC GGG GAC GTG CTC 1298
Thr Ser Asp Tyr Tyr Asp Gly Val Arg Arg Arg Ala Gly Asp Val Leu
335 340 345

ACG GAA GCA CAG ATT AAG GAG TGC CAG GAG CTA GGG GTG CTG GTG GAC 1346
Thr Glu Ala Gln Ile Lys Glu Cys Gln Glu Leu Gly Val Leu Val Asp
350 355 360

AGG GAT GAC CAG GGC GTG CTG CTC CAA ATC TTC ACC AAG CCA GTG GGG 1394
Arg Asp Asp Gln Gly Val Leu Leu Gln Ile Phe Thr Lys Pro Val Gly
365 370 375

GAC AGG CCA ACG CTG TTC TTG GAA ATC ATC CAA AGG ATC GGG TGC ATG 1442
Asp Arg Pro Thr Leu Phe Leu Glu Ile Ile Gln Arg Ile Gly Cys Met
380 385 390

GAG AAG GAT GAG AAG GGG CAA GAA TAC CAA AAG GGT GGC TGC GGC GGG 1490
Glu Lys Asp Glu Lys Gly Gln Glu Tyr Gln Lys Gly Gly Cys Gly Gly
395 400 405 410

TTC GGC AAG GGA AAC TTC TCG CAG CTG TTC AAG TCC ATC GAG GAT TAT 1538
Phe Gly Lys Gly Asn Phe Ser Gln Leu Phe Lys Ser Ile Glu Asp Tyr
415 420 425

GAG AAG TCC CTT GAA GCC AAG CAA GCT GCT GCA GCA GCT GCA GCT CAG 1586
Glu Lys Ser Leu Glu Ala Lys Gln Ala Ala Ala Ala Ala Ala Ala Gln
430 435 440

GGA TCC TAG GACAGTGCTT GGAGACGAGC AACTGCTGTG GCACTTTGTA 1635
Gly Ser

TCATGGAACA GAAATAATGA AGCGTGTTCT TTGTGACACT TGACATGCAA ATGTTTGTGT 1695

TCTGTAACCG TTGAATATAT GGGACGATGC TATGATGGTG TAATAGATGG TAGAGAGGGT 1755

ACAACCCTGA T 1766

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 445 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Met Pro Pro Thr Pro Thr Ala Ala Ala Ala Gly Ala Ala Val Ala Ala
1 5 10 15

Ala Ser Ala Ala Glu Gln Ala Ala Phe Arg Leu Val Gly His Arg Asn
20 25 30

Phe Val Arg Phe Asn Pro Arg Ser Asp Arg Phe His Thr Leu Ala Phe
35 40 45

His His Val Glu Leu Trp Cys Ala Asp Ala Ala Ser Ala Ala Gly Arg
50 55 60

Phe Ser Phe Gly Leu Gly Ala Pro Leu Ala Ala Arg Ser Asp Leu Ser
65 70 75 80

Thr Gly Asn Ser Ala His Ala Ser Leu Leu Leu Arg Ser Gly Ser Leu
85 90 95

Ser Phe Leu Phe Thr Ala Pro Tyr Ala His Gly Ala Asp Ala Ala Thr
100 105 110

Ala Ala Leu Pro Ser Phe Ser Ala Ala Ala Ala Arg Arg Phe Ala Ala
115 120 125

Asp His Gly Leu Ala Val Arg Ala Val Ala Leu Arg Val Ala Asp Ala
130 135 140

Glu Asp Ala Phe Arg Ala Ser Val Ala Ala Gly Ala Arg Pro Ala Phe
145 150 155 160

Gly Pro Val Asp Leu Gly Arg Gly Phe Arg Leu Ala Glu Val Glu Leu
165 170 175

Tyr Gly Asp Val Val Leu Arg Tyr Val Ser Tyr Pro Asp Gly Ala Ala
180 185 190

Gly Glu Pro Phe Leu Pro Gly Phe Glu Gly Val Ala Ser Pro Gly Ala
195 200 205

Ala Asp Tyr Gly Leu Ser Arg Phe Asp His Ile Val Gly Asn Val Pro
210 215 220

Glu Leu Ala Pro Ala Ala Ala Tyr Phe Ala Gly Phe Thr Gly Phe His
225 230 235 240

Glu Phe Ala Glu Phe Thr Thr Glu Asp Val Gly Thr Ala Glu Ser Gly
245 250 255

Leu Asn Ser Met Val Leu Ala Asn Asn Ser Glu Asn Val Leu Leu Pro
260 265 270

Leu Asn Glu Pro Val His Gly Thr Lys Arg Arg Ser Gln Ile Gln Thr
275 280 285

Phe Leu Asp His His Gly Gly Pro Gly Val Gln His Met Ala Leu Ala
290 295 300

Ser Asp Asp Val Leu Arg Thr Leu Arg Glu Met Gln Ala Arg Ser Ala
305 310 315 320

Met Gly Gly Phe Glu Phe Met Ala Pro Pro Thr Ser Asp Tyr Tyr Asp
325 330 335

```
Gly Val Arg Arg Arg Ala Gly Asp Val Leu Thr Glu Ala Gln Ile Lys
            340                 345                 350

Glu Cys Gln Glu Leu Gly Val Leu Val Asp Arg Asp Asp Gln Gly Val
        355                 360                 365

Leu Leu Gln Ile Phe Thr Lys Pro Val Gly Asp Arg Pro Thr Leu Phe
    370                 375                 380

Leu Glu Ile Ile Gln Arg Ile Gly Cys Met Glu Lys Asp Glu Lys Gly
385                 390                 395                 400

Gln Glu Tyr Gln Lys Gly Gly Cys Gly Gly Phe Gly Lys Gly Asn Phe
                405                 410                 415

Ser Gln Leu Phe Lys Ser Ile Glu Asp Tyr Glu Lys Ser Leu Glu Ala
            420                 425                 430

Lys Gln Ala Ala Ala Ala Ala Ala Ala Gln Gly Ser
        435                 440
```

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1356 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Arabidopsis thaliana

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 1..1254

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1..3
(D) OTHER INFORMATION: /standard_name= "translation initiation codon"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1252..1254
(D) OTHER INFORMATION: /standard_name= "translation termination codon"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```
ATG TCC AAG TTC GTA AGA AAG AAT CCA AAG TCT GAT AAA TTC AAG GTT      48
Met Ser Lys Phe Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val
  1               5                  10                  15

AAG CGC TTC CAT CAC ATC GAG TTC TGG TGC GGC GAC GCA ACC AAC GTC      96
Lys Arg Phe His His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val
             20                  25                  30
```

```
GCT CGT CGC TTC TCC TGG GGT CTG GGG ATG AGA TTC TCC GCC AAA TCC      144
Ala Arg Arg Phe Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser
         35                  40                  45

GAT CTT TCC ACC GGA AAC ATG GTT CAC GCC TCT TAC CTA CTC ACC TCC      192
Asp Leu Ser Thr Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser
     50                  55                  60

GGT GAC CTC CGA TTC CTT TTC ACT GCT CCT TAC TCT CCG TCT CTC TCC      240
Gly Asp Leu Arg Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser
 65                  70                  75                  80

GCC GGA GAG ATT AAA CCG ACA ACC ACA GCT TCT ATC CCA AGT TTC GAT      288
Ala Gly Glu Ile Lys Pro Thr Thr Thr Ala Ser Ile Pro Ser Phe Asp
                 85                  90                  95

CAC GGC TCT TGT CGT TCC TTC TTC TCT TCA CAT GGT CTC GGT GTT AGA      336
His Gly Ser Cys Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg
            100                 105                 110

GCC GTT GCG ATT GAA GTA GAA GAC GCA GAG TCA GCT TTC TCC ATC AGT      384
Ala Val Ala Ile Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser
        115                 120                 125

GTA GCT AAT GGC GCT ATT CCT TCG TCG CCT CCT ATC GTC CTC AAT GAA      432
Val Ala Asn Gly Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu
    130                 135                 140

GCA GTT ACG ATC GCT GAG GTT AAA CTA TAC GGC GAT GTT GTT CTC CGA      480
Ala Val Thr Ile Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg
145                 150                 155                 160

TAT GTT AGT TAC AAA GCA GAA GAT ACC GAA AAA TCC GAA TTC TTG CCA      528
Tyr Val Ser Tyr Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro
                165                 170                 175

GGG TTC GAG CGT GTA GAG GAT GCG TCG TCG TTC CCA TTG GAT TAT GGT      576
Gly Phe Glu Arg Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly
            180                 185                 190

ATC CGG CGG CTT GAC CAC GCC GTG GGA AAC GTT CCT GAG CTT GGT CCG      624
Ile Arg Arg Leu Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro
        195                 200                 205

GCT TTA ACT TAT GTA GCG GGG TTC ACT GGT TTT CAC CAA TTC GCA GAG      672
Ala Leu Thr Tyr Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu
    210                 215                 220

TTC ACA GCA GAC GAC GTT GGA ACC GCC GAG AGC GGT TTA AAT TCA GCG      720
Phe Thr Ala Asp Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala
225                 230                 235                 240

GTC CTG GCT AGC AAT GAT GAA ATG GTT CTT CTA CCG ATT AAC GAG CCA      768
Val Leu Ala Ser Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro
                245                 250                 255

GTG CAC GGA ACA AAG AGG AAG AGT CAG ATT CAG ACG TAT TTG GAA CAT      816
Val His Gly Thr Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His
            260                 265                 270

AAC GAA GGC GCA GGG CTA CAA CAT CTG GCT CTG ATG AGT GAA GAC ATA      864
Asn Glu Gly Ala Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile
        275                 280                 285
```

```
TTC AGG ACC CTG AGA GAG ATG AGG AAG AGG AGC AGT ATT GGA GGA TTC      912
Phe Arg Thr Leu Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe
    290                 295                 300

GAC TTC ATG CCT TCT CCT CCG CCT ACT TAC TAC CAG AAT CTC AAG AAA      960
Asp Phe Met Pro Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys
305                 310                 315                 320

CGG GTC GGC GAC GTG CTC AGC GAT GAT CAG ATC AAG GAG TGT GAG GAA     1008
Arg Val Gly Asp Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu
                325                 330                 335

TTA GGG ATT CTT GTA GAC AGA GAT GAT CAA GGG ACG TTG CTT CAA ATC     1056
Leu Gly Ile Leu Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile
            340                 345                 350

TTC ACA AAA CCA CTA GGT GAC AGG CCG ACG ATA TTT ATA GAG ATA ATC     1104
Phe Thr Lys Pro Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu Ile Ile
        355                 360                 365

CAG AGA GTA GGA TGC ATG ATG AAA GAT GAG GAA GGG AAG GCT TAC CAG     1152
Gln Arg Val Gly Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln
    370                 375                 380

AGT GGA GGA TGT GGT GGT TTT GGC AAA GGC AAT TTC TCT GAG CTC TTC     1200
Ser Gly Gly Cys Gly Gly Phe Gly Lys Gly Asn Phe Ser Glu Leu Phe
385                 390                 395                 400

AAG TCC ATT GAA GAA TAC GAA AAG ACT CTT GAA GCC AAA CAG TTA GTG     1248
Lys Ser Ile Glu Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val
                405                 410                 415

GGA TGA ACAAGAAGAA GAACCAACTA AAGGATTGTG TAATTAATGT AAAACTGTTT      1304
Gly  *

TATCTTATCA AAACAATGTA TACAACATCT CATTTAAAAA CGAGATCAAT CC           1356
```

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 418 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```
Met Ser Lys Phe Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val
 1               5                  10                  15

Lys Arg Phe His His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val
            20                  25                  30

Ala Arg Arg Phe Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser
        35                  40                  45

Asp Leu Ser Thr Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser
    50                  55                  60

Gly Asp Leu Arg Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser
65                  70                  75                  80

Ala Gly Glu Ile Lys Pro Thr Thr Thr Ala Ser Ile Pro Ser Phe Asp
                85                  90                  95
```

His Gly Ser Cys Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg
100 105 110

Ala Val Ala Ile Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser
115 120 125

Val Ala Asn Gly Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu
130 135 140

Ala Val Thr Ile Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg
145 150 155 160

Tyr Val Ser Tyr Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro
165 170 175

Gly Phe Glu Arg Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly
180 185 190

Ile Arg Arg Leu Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro
195 200 205

Ala Leu Thr Tyr Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu
210 215 220

Phe Thr Ala Asp Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala
225 230 235 240

Val Leu Ala Ser Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro
245 250 255

Val His Gly Thr Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His
260 265 270

Asn Glu Gly Ala Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile
275 280 285

Phe Arg Thr Leu Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe
290 295 300

Asp Phe Met Pro Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys
305 310 315 320

Arg Val Gly Asp Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu
325 330 335

Leu Gly Ile Leu Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile
340 345 350

Phe Thr Lys Pro Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu Ile Ile
355 360 365

Gln Arg Val Gly Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln
370 375 380

Ser Gly Gly Cys Gly Gly Phe Gly Lys Gly Asn Phe Ser Glu Leu Phe
385 390 395 400

Lys Ser Ile Glu Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val
405 410 415

Gly

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1448 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Arabidopsis thaliana

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 9..1346

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 9..11
(D) OTHER INFORMATION: /standard_name= "translation initiation codon"

(ix) FEATURE:
(A) NAME/KEY: misc_feature
(B) LOCATION: 1344..1346
(D) OTHER INFORMATION: /standard_name= "translation termination codon"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

```
TGAAATCA ATG GGC CAC CAA AAC GCC GCC GTT TCA GAG AAT CAA AAC CAT      50
         Met Gly His Gln Asn Ala Ala Val Ser Glu Asn Gln Asn His
           1               5                  10

GAT GAC GGC GCT GCG TCG TCG CCG GGA TTC AAG CTC GTC GGA TTT TCC       98
Asp Asp Gly Ala Ala Ser Ser Pro Gly Phe Lys Leu Val Gly Phe Ser
 15                  20                  25                  30

AAG TTC GTA AGA AAG AAT CCA AAG TCT GAT AAA TTC AAG GTT AAG CGC      146
Lys Phe Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val Lys Arg
                 35                  40                  45

TTC CAT CAC ATC GAG TTC TGG TGC GGC GAC GCA ACC AAC GTC GCT CGT      194
Phe His His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val Ala Arg
             50                  55                  60

CGC TTC TCC TGG GGT CTG GGG ATG AGA TTC TCC GCC AAA TCC GAT CTT      242
Arg Phe Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser Asp Leu
         65                  70                  75

TCC ACC GGA AAC ATG GTT CAC GCC TCT TAC CTA CTC ACC TCC GGT GAC      290
Ser Thr Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser Gly Asp
     80                  85                  90

CTC CGA TTC CTT TTC ACT GCT CCT TAC TCT CCG TCT CTC TCC GCC GGA      338
Leu Arg Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser Ala Gly
 95                 100                 105                 110

GAG ATT AAA CCG ACA ACC ACA GCT TCT ATC CCA AGT TTC GAT CAC GGC      386
Glu Ile Lys Pro Thr Thr Thr Ala Ser Ile Pro Ser Phe Asp His Gly
                115                 120                 125
```

TCT TGT CGT TCC TTC TTC TCT TCA CAT GGT CTC GGT GTT AGA GCC GTT 434
Ser Cys Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg Ala Val
130 135 140

GCG ATT GAA GTA GAA GAC GCA GAG TCA GCT TTC TCC ATC AGT GTA GCT 482
Ala Ile Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser Val Ala
145 150 155

AAT GGC GCT ATT CCT TCG TCG CCT CCT ATC GTC CTC AAT GAA GCA GTT 530
Asn Gly Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu Ala Val
160 165 170

ACG ATC GCT GAG GTT AAA CTA TAC GGC GAT GTT GTT CTC CGA TAT GTT 578
Thr Ile Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg Tyr Val
175 180 185 190

AGT TAC AAA GCA GAA GAT ACC GAA AAA TCC GAA TTC TTG CCA GGG TTC 626
Ser Tyr Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro Gly Phe
195 200 205

GAG CGT GTA GAG GAT GCG TCG TCG TTC CCA TTG GAT TAT GGT ATC CGG 674
Glu Arg Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly Ile Arg
210 215 220

CGG CTT GAC CAC GCC GTG GGA AAC GTT CCT GAG CTT GGT CCG GCT TTA 722
Arg Leu Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro Ala Leu
225 230 235

ACT TAT GTA GCG GGG TTC ACT GGT TTT CAC CAA TTC GCA GAG TTC ACA 770
Thr Tyr Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu Phe Thr
240 245 250

GCA GAC GAC GTT GGA ACC GCC GAG AGC GGT TTA AAT TCA GCG GTC CTG 818
Ala Asp Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala Val Leu
255 260 265 270

GCT AGC AAT GAT GAA ATG GTT CTT CTA CCG ATT AAC GAG CCA GTG CAC 866
Ala Ser Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro Val His
275 280 285

GGA ACA AAG AGG AAG AGT CAG ATT CAG ACG TAT TTG GAA CAT AAC GAA 914
Gly Thr Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His Asn Glu
290 295 300

GGC GCA GGG CTA CAA CAT CTG GCT CTG ATG AGT GAA GAC ATA TTC AGG 962
Gly Ala Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile Phe Arg
305 310 315

ACC CTG AGA GAG ATG AGG AAG AGG AGC AGT ATT GGA GGA TTC GAC TTC 1010
Thr Leu Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe Asp Phe
320 325 330

ATG CCT TCT CCT CCG CCT ACT TAC TAC CAG AAT CTC AAG AAA CGG GTC 1058
Met Pro Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys Arg Val
335 340 345 350

GGC GAC GTG CTC AGC GAT GAT CAG ATC AAG GAG TGT GAG GAA TTA GGG 1106
Gly Asp Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu Leu Gly
355 360 365

ATT CTT GTA GAC AGA GAT GAT CAA GGG ACG TTG CTT CAA ATC TTC ACA 1154
Ile Leu Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile Phe Thr
370 375 380

```
AAA CCA CTA GGT GAC AGG CCG ACG ATA TTT ATA GAG ATA ATC CAG AGA    1202
Lys Pro Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu Ile Ile Gln Arg
        385                 390                 395

GTA GGA TGC ATG ATG AAA GAT GAG GAA GGG AAG GCT TAC CAG AGT GGA    1250
Val Gly Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln Ser Gly
    400                 405                 410

GGA TGT GGT GGT TTT GGC AAA GGC AAT TTC TCT GAG CTC TTC AAG TCC    1298
Gly Cys Gly Gly Phe Gly Lys Gly Asn Phe Ser Glu Leu Phe Lys Ser
415                 420                 425                 430

ATT GAA GAA TAC GAA AAG ACT CTT GAA GCC AAA CAG TTA GTG GGA TGA    1346
Ile Glu Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val Gly  *
                435                 440                 445

ACAAGAAGAA GAACCAACTA AAGGATTGTG TAATTAATGT AAAACTGTTT TATCTTATCA 1406

AAACAATGTA TACAACATCT CATTTAAAAA CGAGATCAAT CC                    1448
```

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 446 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Met Gly His Gln Asn Ala Ala Val Ser Glu Asn Gln Asn His Asp Asp
  1               5                  10                  15

Gly Ala Ala Ser Ser Pro Gly Phe Lys Leu Val Gly Phe Ser Lys Phe
             20                  25                  30

Val Arg Lys Asn Pro Lys Ser Asp Lys Phe Lys Val Lys Arg Phe His
         35                  40                  45

His Ile Glu Phe Trp Cys Gly Asp Ala Thr Asn Val Ala Arg Arg Phe
     50                  55                  60

Ser Trp Gly Leu Gly Met Arg Phe Ser Ala Lys Ser Asp Leu Ser Thr
 65                  70                  75                  80

Gly Asn Met Val His Ala Ser Tyr Leu Leu Thr Ser Gly Asp Leu Arg
                 85                  90                  95

Phe Leu Phe Thr Ala Pro Tyr Ser Pro Ser Leu Ser Ala Gly Glu Ile
            100                 105                 110

Lys Pro Thr Thr Thr Ala Ser Ile Pro Ser Phe Asp His Gly Ser Cys
        115                 120                 125

Arg Ser Phe Phe Ser Ser His Gly Leu Gly Val Arg Ala Val Ala Ile
    130                 135                 140

Glu Val Glu Asp Ala Glu Ser Ala Phe Ser Ile Ser Val Ala Asn Gly
145                 150                 155                 160

Ala Ile Pro Ser Ser Pro Pro Ile Val Leu Asn Glu Ala Val Thr Ile
                165                 170                 175

Ala Glu Val Lys Leu Tyr Gly Asp Val Val Leu Arg Tyr Val Ser Tyr
            180                 185                 190
```

```
Lys Ala Glu Asp Thr Glu Lys Ser Glu Phe Leu Pro Gly Phe Glu Arg
        195                 200                 205

Val Glu Asp Ala Ser Ser Phe Pro Leu Asp Tyr Gly Ile Arg Arg Leu
    210                 215                 220

Asp His Ala Val Gly Asn Val Pro Glu Leu Gly Pro Ala Leu Thr Tyr
225                 230                 235                 240

Val Ala Gly Phe Thr Gly Phe His Gln Phe Ala Glu Phe Thr Ala Asp
                245                 250                 255

Asp Val Gly Thr Ala Glu Ser Gly Leu Asn Ser Ala Val Leu Ala Ser
            260                 265                 270

Asn Asp Glu Met Val Leu Leu Pro Ile Asn Glu Pro Val His Gly Thr
        275                 280                 285

Lys Arg Lys Ser Gln Ile Gln Thr Tyr Leu Glu His Asn Glu Gly Ala
    290                 295                 300

Gly Leu Gln His Leu Ala Leu Met Ser Glu Asp Ile Phe Arg Thr Leu
305                 310                 315                 320

Arg Glu Met Arg Lys Arg Ser Ser Ile Gly Gly Phe Asp Phe Met Pro
                325                 330                 335

Ser Pro Pro Pro Thr Tyr Tyr Gln Asn Leu Lys Lys Arg Val Gly Asp
            340                 345                 350

Val Leu Ser Asp Asp Gln Ile Lys Glu Cys Glu Glu Leu Gly Ile Leu
        355                 360                 365

Val Asp Arg Asp Asp Gln Gly Thr Leu Leu Gln Ile Phe Thr Lys Pro
    370                 375                 380

Leu Gly Asp Arg Pro Thr Ile Phe Ile Glu Ile Ile Gln Arg Val Gly
385                 390                 395                 400

Cys Met Met Lys Asp Glu Glu Gly Lys Ala Tyr Gln Ser Gly Gly Cys
                405                 410                 415

Gly Gly Phe Gly Lys Gly Asn Phe Ser Glu Leu Phe Lys Ser Ile Glu
            420                 425                 430

Glu Tyr Glu Lys Thr Leu Glu Ala Lys Gln Leu Val Gly
        435                 440                 445
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 513 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Vernonia galamenensis

(vii) IMMEDIATE SOURCE:
(B) CLONE: vs1.pk0015.b2

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
CCACACCGAT TGCCGGAACT TCACCGCCTC TCACGGCCTT GCAGTCCGAG CAATCGCCAT   60
TGAAGTCGAT GACGCCGAAT TAGCTTTCTC CGTCAGCGTC TCTCACGGCG CTAAACCCTC  120
CGCTGCTCCT GTAACCCTTG GAAACAACGA CGTCGTATTG TCTGAAGTTA AGCTTTACGG  180
CGATGTCGCT TTCCGGTACA TAAGTTACAA AAATCCGAAC TATACATCTT CCTTTTTGCC  240
CGGGTTCGAG CCCGTTGAAA AGACGTCGTC GTTTTATGAC CTTGACTACG GTATCCGCCG  300
TTTGGACCAC GCCGTAGGNA ACGTCCCTGA GCTTGCTTCG GCAGTGGACT ACGTGAAATC  360
ATTCACCGGA TTCCATGAGT TCGCCGAATT CACCGCGGAG GACCTCGGGA CGAGCGAGAG  420
GGAACTGAAT TCGGTCGTTT TAGCTTGCAA CAGTGAGATG GTCTTGATTC CGATGAACGA  480
GCCGGTGTAC GGAANAAAAG GAAGNAGCCA GAT                               513
```

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 6, line 1

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*

12301 Parklawn Drive
Rockville, Maryland 20852
US

Date of deposit	Accession Number
25 June 1996 (25.06.96)	98083

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ■

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 6, line 1.

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
12301 Parklawn Drive
Rockville, Maryland 20852
US

Date of deposit	Accession Number
25 June 1996 (25.06.96)	97622

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ■

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 6, line 1

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution
AMERICAN TYPE CULTURE COLLECTION

Address of depositary institution *(including postal code and country)*
12301 Parklawn Drive
Rockville, Maryland 20852
US

Date of deposit	Accession Number
12 June 1997	209120

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the European patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

CLAIMS

1. An isolated nucleic acid fragment encoding a plant *p*-hydroxyphenylpyruvate dioxygenase enzyme, the fragment comprising a nucleotide sequence selected from the group consisting of

nucleotide sequences encoding a polypeptide comprising the amino acid sequences set forth in SEQ ID NO:3, SEQ ID NO:11, SEQ ID NO:13, and SEQ ID NO:15 and

modified nucleotide sequences essentially similar to the nucleotide sequences of SEQ ID NO:2, SEQ ID NO 10, SEQ ID NO:12 and SEQ ID NO:14 containing deletions, insertions, or substitutions in the sequence that do not affect the functional properties of the encoded protein.

2. An isolated nucleic acid fragment encoding a plant *p*-hydroxyphenylpyruvate dioxygenase enzyme, the fragment comprising a nucleotide sequence as set forth in SEQ ID NO:14.

3. A chimeric gene comprising the nucleic acid fragment of Claims 1 or 2 operably linked to at least one suitable regulatory sequence.

4. The chimeric gene of Claim 3 wherein at least one suitable regulatory sequence directs gene expression in a microorganism.

5. The chimeric gene of Claim 3 wherein the at least one suitable regulatory sequence directs gene expression in a plant.

6. A plasmid vector comprising the nucleic acid fragment of Claims 1 or 2 operablylinked to at least one suitable regulatory sequence.

7. A transformed host cell comprising a host cell and the plasmid vector of Claim 6.

8. The transformed host cell of Claim 7 wherein the host cell is derived from a plant or is a microorganism.

9. The transformed host cell of Claim 8 wherein the microorganism is *E. coli*.

10. A transformed plant tolerant to contact with at least one compound that inhibits the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme in a non-transformed plant, the transformed plant comprising the chimeric gene of Claim 3 and a host plant.

11. The transformed plant of Claim 10 wherein the host plant is a cereal crop plant.

12. A method to identify a compound useful for its ability to inhibit the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme comprising:

(a) transforming a host cell with the plasmid vector of Claim 6;

(b) facilitating expression of the nucleic acid fragment encoding the plant *p*-hydroxyphenylpyruvate dioxygenase enzyme:

(c) contacting the expressed enzyme from step (b) with a test compound: and

(d) evaluating the capacity of the test compound to inhibit the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme.

13. The method of Claim 12 wherein evaluating the capacity of the test compound to inhibit the rate of the reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme is accomplished by measuring oxygen utilization, carbon dioxide release, homogentisate production, loss of *p*-hydroxyphenylpyruvate or maleylacetoacetate production.

14. The method of Claim 12 wherein the transformed host cell is an *E. coli* that comprises a chimeric gene encoding a plant *p*-hydroxyphenylpyruvate dioxygenase enzyme.

15. A compound that inhibits the activity of a plant *p*-hydroxyphenylpyruvate dioxygenase enzyme, the compound identified by the method of Claim 14.

16. A method for imparting tolerance to a plant to at least one compound that inhibits the rate of reaction of *p*-hydroxyphenylpyruvate dioxygenase enzyme comprising:

(a) transforming a host plant cell with a chimeric gene comprising a nucleic acid fragment encoding plant *p*-hydroxyphenylpyruvate dioxygenase, and

(b) expressing the chimeric gene in an amount effective to render the transformed plant substantially tolerant to the at least one compound that inhibits the rate of reaction of *p*-hydroxyphenylpyruvate dioxygenase.

17. A method for the microbial production of active plant *p*-hydroxyphenylpyruvate dioxygenase enzyme comprising:

(a) stably transforming a microorganism with the chimeric gene of Claim 4 encoding the plant p-hydroxyphenylpyruvate dioxygenase;

(b) facilitating expression by the chimeric gene for a suitable period; and

(c) recovering active plant *p*-hydroxyphenylpyruvate dioxygenase enzyme.

18. A method to overexpress *p*-hydroxyphenylpyruvate dioxygenase enzyme in a plant comprising:

(a) stably transforming a host plant cell with a chimeric DNA molecule comprising at least one copy of a suitable promoter to drive expression of an associated coding sequence in a plant cell operably linked to at least one copy of a homologous or heterologous coding sequence encoding *p*-hydroxyphenyl-pyruvate dioxygenase; and

(b) growing the transformed host plant cell of step (a).

19. The method of Claim 18 wherein the chimeric DNA molecule is the chimeric gene of Claim 5.

20. An isolated nucleic acid fragment comprising a member selected from the group consisting of:

(a) an isolated nucleic acid fragment as set forth in SEQ ID NO:16;

(b) an isolated nucleic acid fragment that is essentially similar to an isolated nucleic acid fragment as set forth in SEQ ID NO:16; and

(c) an isolated nucleic acid fragment that is complementary to (a) or (b).

FIG. 1

1 CAAGAAACGNGTCGNCGACGTGCTCAGCGATGATCAGATCAAGGAGTGTGAGGAATTAGG

61 GATTCTTNTAGACAGAGATGATCAAGGGACGTTNCTTCAAATCTNCACAAAACCACTAGG

121 TGACAGGCCGACGNTATTTATAGAGATAATCCAGAGNGTAGGATGCATGATGAAAGATGT

181 GGAAGGGANGGCTTACCAGAGTGGAGNATNTNGTGGTTTTGGCAAAGGCAATT

FIG.2

1 TGAAATCA**ATG**GGCCACCAAAACGCCGCCGTTTCAGAGAATCAAAACCATGATGACGGCG

61 CTGCGTCGTCGCCGGGATTCAAGCTCGTCGGATTTTCCAAGTTCGTAAGAAAGAATCCAA

121 AGTCTGATAAATTCAAGGTTAAGCGCTTCCATCACATCGAGTTCTGGTGCGGGGACGCAA
*Eco*47III

181 CCAACGTCGCTCGTCGCTTCTCCTGGGGTCTGGGGATGAGATTCTCCGCCAAATCCGATC

241 TTTCCACCGGAAACATGGTTCACGCCTCTTACCTACTCACCTCCGGTGAACTCCGATTCC

301 TTTTCACTGCTCCTTACTCTCCGTCTCTCTCCGGCGGAGAGATTAAACCGACAACCACAG

361 GTTCTATCCCAAGTTTCGATCACGGGTCTTGTCGGTCCTTCTTCTCTTCACATGGTCTCG

421 GTGTTAGACCCGTTGCGATTGAAGTAGAAGACGCGGAGTCAGCTTTCTCCATCAGTGTAG

481 CTAATGGCGCTATTCCTTCGTCGCCTCCTATCGTCCTCAATGAAGCAGTTACGATCGCTG

541 AGGTTAAACTATACGGCGATGTTGTTCTCCGATATGTTAGTTACAAAGCAGAAGATACCG

601 AAAAATCCGAATTCTTGCCAGGGTTCGAGCGTGTAGAGGATGCGTCGTCGTTCCCATTGG
*Eco*RI

661 ATTATGGTATCCGGCGGCTTGACCACGCCGTGGGAAACGTTCCTGAGCTTGGTCCGGCTT

721 TAACTTATGTAGCGGGGTTCACTGGTTTTCACCAATTCGCAGAGTTCACAGCAGACGACG

781 TTGGAACCGCCGAGAGCGGTTTAAATTCAGCGGTCCTGGCTAGCAATGATGAAATGGTTC
*Nhe*I

841 TTCTACCGATTAACGAGCCAGTGCACGGAACAAAGAGGAAGAGTCAGATTCAGACGTATT

901 TGGAACATAACGAAGGCGCAGGGCTACAACATCTGGCTCTGATGAGTGAAGACATATTCA

961 GGACCCTGAGAGAGATGAGGAAGAGGAGCAGTATTGGAGGATTCGACTTCATGCCTTCTC

1021 CTCCGCCTACTTACTACCAGAATCTCAAGAAACGGGTCGGCGACGTGCTCAGCGATGATC

1081 AGATCAAGGAGTGTGAGGAATTAGGGATTCTTGTAGACAGAGATGATCAAGGGACGTTGC

1141 TTCAAATCTTCACAAAACCACTAGGTGACAGGCCGACGATATTTATAGAGATAATCCAGA

1201 GAGTAGGATGCATGATGAAAGATGAGGAAGGGAAGGCTTACCAGAGTGGAGGATGTGGTG

1261 GTTTTGCCAAAGGCAATTTCTCTGAGCTCTTCAAGTCCATTGAAGAATACGAAAAGACTC

1321 TTGAAGCCAAACAGTTAGTGGGA**TGA**ACAAGAAGAAGAACCAACTAAAGGATTGTGTAAT

1381 TAATGTAAAACTGTTTTATCTTATCAAAACAATGTATACAACATCTCATTTAAAAACGAG

1441 ATCAATCC

3/6

FIG.3A

```
             1                                                   50
 Arabidopsis  MGHQNAAVS ENQNHDDGAA SSPGFKLVGF SKFVRKNPKS DKFKVKRFHH
        Corn MPPTPTAAAA GAAVAAASAA EQAAFRLVGH RNFVRFNPRS DRFHTLAFHH
         Rat                                     YWDKGPKP ERGRFLHFHS
       Mouse                               M TTYNNKGPKP ERGRFLHFHS
       Human                               M TTYSDKGAKP ERGRFLHFHS
         Pig                               M TSYSDKGEKP ERGRFLHFHS
                                                                 **

             51                                                 100
 Arabidopsis IEFWCGDATN VARRFSWGLG MRFSAKSDLS TGNMVHASYL LTSGDLRFLF
        Corn VELWCADAAS AAGRFSFGLG APLAARSDLS TGNSAHASLL LRSGSLSFLF
         Rat VTFWVGNAKQ AASFYCNKMG FEPLAYKGLE TGSREVVSHV IKQGKIVFVL
       Mouse VTFWVGNAKQ AASFYCNKMG FEPLAYRGLE TGSREVVSHV IKRGKIVFVL
       Human VTFWVGNAKQ AASFYCSKMG FEPLAYRGLE TGSREVVSHV IKQGKIVFVL
         Pig VTFWVGNAKQ AASYYCSKIG FEPLAYKGLE TGSREVVSHV VKQDKIVFVF
                *   *    *       *     *   *  **     *          *

             101                                                150
 Arabidopsis TAPYSPSLSA GEIKPTTTAS IPSFDHGSCR SFFSSHGLGV RAVAIEVEDA
        Corn TAPYAHGADA ......ATAA LPSFSAAAAR RFAADHGLAV RAVALRVADA
         Rat CSALNPW... .......... .....NKEMG DHLVKHGDGV KDIAFEVEDC
       Mouse CSALNPW... .......... .....NKEMG DHLVKHGDGV KDIAFEVEDC
       Human SSALNPW... .......... .....NKEMG DHLVKHGDGV KDIAFEVEDC
         Pig SSALNPW... .......... .....NKEMG DHLVKHGDGV KDIAFEVEDC
                                                   **  *    *  * *

             151                                                200
 Arabidopsis ESAFSISVAN GAIPSSPPIV LNEAVTIAEV KLYGDVVLRY VSYKAEDTEK
        Corn EDAFRASVAA GARPAFGPVD LGRGFRLAEV ELYGDVVLRY VSY.PDGAAG
         Rat EHIVQKARER GAKIVREPWV EEDKFGKVKF AVLQTYGDTT HTLVEKINYT
       Mouse DHIVQKARER GAKIVREPWV EQDKFGKVKF AVLQTYGDTT HTLVEKINYT
       Human DYIVQKARER GAKIMREPWV EQDKFGKVKF AVLQTYGDTT HTLVEKMNYI
         Pig DYIVQKARER GAIIVREPWI EQDKFGKVKF AVLQTFGDTT HTLVEKMNYT
                        **     *

             201                                                250
 Arabidopsis SEFLPGFER. ..VEDASSFP LDYGIRRLDH AVGNVP..EL GPALTYVAGF
        Corn EPFLPGFEG. ..V..ASPGA ADYGLSRFDH IVGNVP..EL APAAAYFAGF
         Rat GRFLPGFEAP TYKDTLLPKL PSCNLEIIDH IVGNQPDQEM ESASEWYLKN
       Mouse GRFLPGFEAP TYKDTLLPKL PRCNLEIIDH IVGNQPDQEM QSASEWYLKN
       Human GQFLPGYEPP AFMDPLLPKL PKCSLEMIDH IVGNQPDQEM VSASEWYLKN
         Pig GCFLPGFEAP TFTDPLLSKL PKCGLEIIDH IVGNQPDQEM ESASQWYMRN
               **** *                      **  *** *  *    *

             251                                                300
 Arabidopsis TGFHQFAEFT ADDVGTAESG LNSAVLASND EMVLLPINEP VHGTKRKSQI
        Corn TGFHEFAEFT TEDVGTAESG LNSMVLANNS ENVLLPLNEP VHGTKRRSQI
         Rat LQFHRFWSVD DTQVHTEYSS LRSIVVANYE ESIKMPINEP APG.RKKSQI
       Mouse LQFHRFWSVD DTQVHTEYSS LRSIVVTNYE ESIKMPINEP APG.RKKSQI
       Human LQFHRFWSVD DTQVHTEYSS LRSIVVANYE ESIKMPINEP APG.KKKSQI
         Pig LQFHRFWSVD DTQIHTEYSA LRSVVMANYE ESIKMPINEP APG.KKKSQI
               ** *          *  *  * * *      *    * ***   *    ***
```

4/6

FIG.3B

```
             301                                                   350
Arabidopsis  QTYLEHNEGA GLQHLALMSE DIFRTLREMR KRSSIGGFDF MPSPPPTYYQ
       Corn  QTFLDHHGGP GVQHMALASD DVLRTLREMQ ARSAMGGFEF MAPPTSDYYD
        Rat  QEYVDYNGGA GVQHIALRTE DIITTIRHLR ER....GMEF LAVP.SSYYR
      Mouse  QEYVDYNGGA GVQHIALKTE DIITAIRHLR ER....GTEF LAAP.SSYYK
      Human  QEYVDYNGGA GVQHIALKTE DIITAIRHLR ER....GLEF LSVP.STYYK
        Pig  QEYVDYNGGA GVQHIALKTE DIITAIRSLR ER....GVEF LAVP.FTYYK
             .       .  . .. ..    .     .     .    .  .    .   ..

             351                                                   400
Arabidopsis  NLKK..RVGD VLSDDQIKEC EELGILVDRD DQGTLLQIFT KPLGDRPTIF
       Corn  GVRR..RAGD VLTEAQIKEC QELGVLVDRD DQGVLLQIFT KPVGDRPTLF
        Rat  LLRENLKTSK IQVKENMDVL EELKILVDYD EKGYLLQIFT KPMQDRPTLF
      Mouse  LLRENLKSAK IQVKESMDVL EELHILVDYD EKGYLLQIFT KPMQDRPTLF
      Human  QLREKLKTAK IKVKENIDAL EELKILVDYD EKGYLLQIFT KPVQDRPTLF
        Pig  QLQEKLKSAK IRVKESIDVL EELKILVDYD EKGYLLQIFT KPMQDRPTVF
                                    **  *** *    * ****** **  **** *

             401                                                   450
Arabidopsis  IEIIQRVGCM MKDEEGKAYQ SGGCGGFGKG NFSELFKSIE EYEKTLEAKQ
       Corn  LEIIQRIGCM EKDEKGQEYQ KGGCGGFGKG NFSQLFKSIE DYEKSLEAKQ
        Rat  LEVIQRHNHQ .......... .....GFGAG NFNSLFKAFE E.EQALRG
      Mouse  LEVIQRHNHQ .......... .....GFGAG NFNSLFKAFE E.EQALRGNL
      Human  LEVIQRHNHQ .......... .....GFGAG NFNSLFKAFE E.EQNLRGNL
        Pig  LEVIQRNNHQ .......... .....GFGAG NFNSLFKAFE E.EQELRGNL
              * ***                     ***   **   ***  *    *  *

             451        462
Arabidopsis  LVG                      (Seq. I.D. No. 15)
       Corn  AAAAAAAQGS               (Seq. I.D. No. 11)
        Rat                           (Seq. I.D. No.  9)
      Mouse  TDLEPNGVRS GM            (Seq. I.D. No.  8)
      Human  TNMETNGVVP GM            (Seq. I.D. No.  6)
        Pig  TDTDPNGVPF RL            (Seq. I.D. No.  7)
```

FIG.4

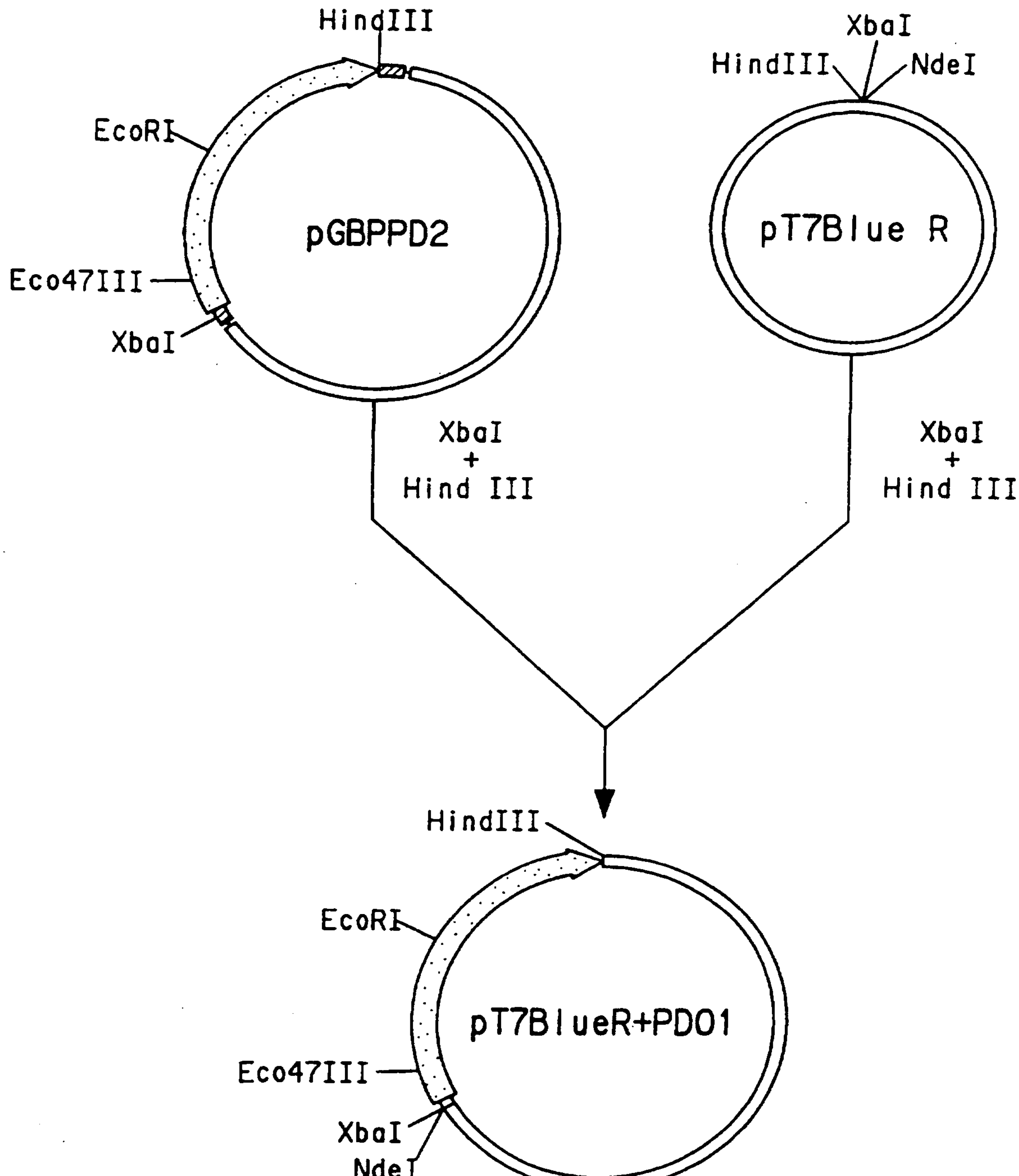
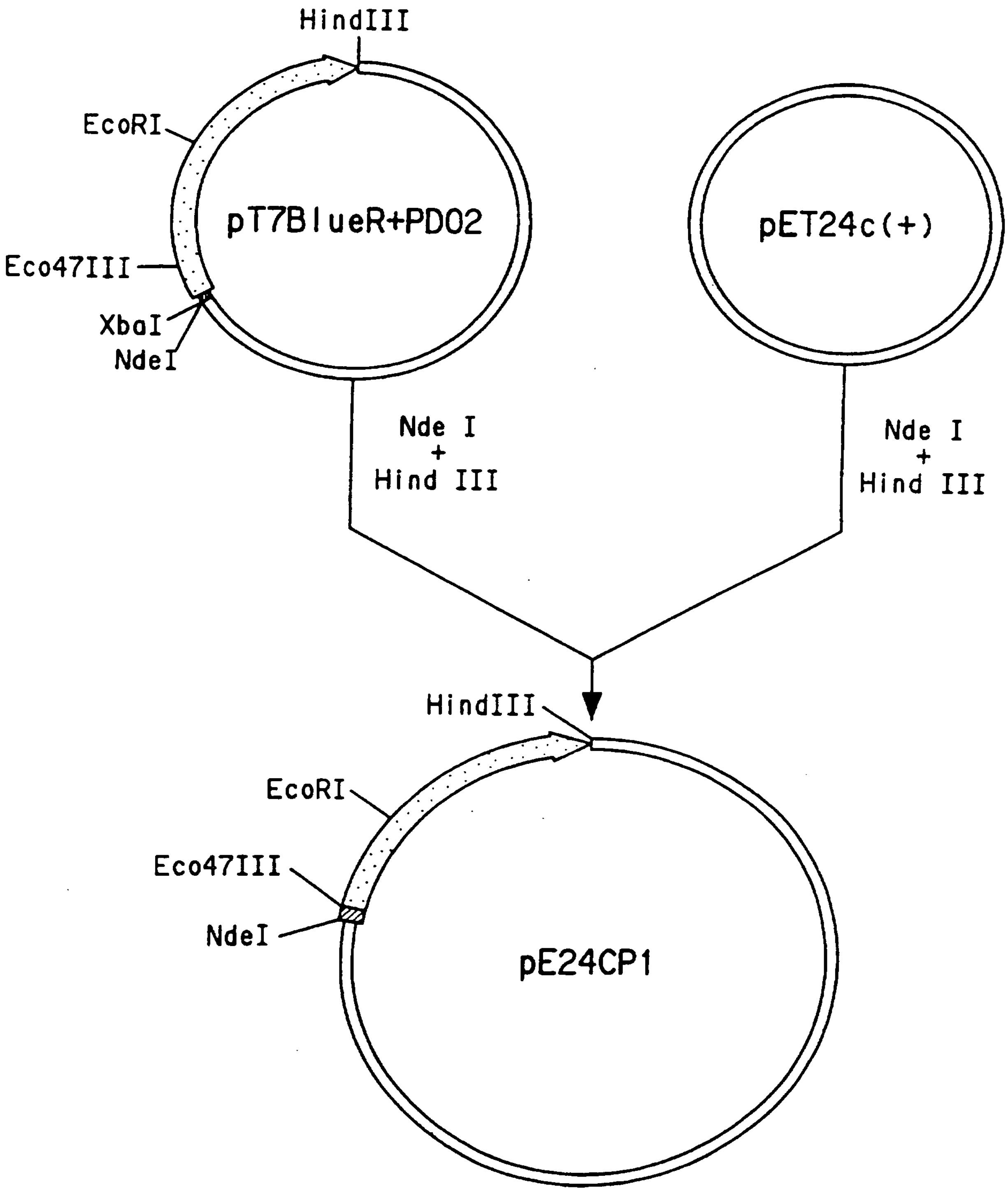

HindIII
EcoRI
pGBPPD2
Eco47III
XbaI
XbaI
HindIII
NdeI
pT7Blue R
XbaI
+
Hind III
XbaI
+
Hind III
HindIII
EcoRI
pT7BlueR+PD01
Eco47III
XbaI
NdeI

FIG.5


HindIII
EcoRI
pT7BlueR+PD02
Eco47III
XbaI
NdeI
pET24c(+)
Nde I
+
Hind III
Nde I
+
Hind III
HindIII
EcoRI
Eco47III
NdeI
pE24CP1

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/11295

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/53 C12N15/82 C12Q1/26 C12Q1/02 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEWMAN, T., ET AL.: "2960 Arabidopsis thaliana cDNA clone 91B13T7" EMBL SEQUENCE DATABASE, REL. 40, 16-JUN-1994, ACCESSION NO. T20952, XP002028637 see sequence ---	1,2
X	NEWMAN, T., ET AL.: "20804 Arabidopsis thaliana cDNA clone 231K20T7" EMBL SEQUENCE DATABASE, REL.47, 8-MAR-1996, ACCESSION NO. N65764, XP002029449 see sequence ---	1,2

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

4

Date of the actual completion of the international search	Date of mailing of the international search report
26 September 1997	07.10.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Maddox, A

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SCHULZ A ET AL: "SC-0051, A 2-BENZOYL-CYCLOHEXANE-1,3-DIONE BLEACHING HERBICIDE, IS APOTENT INHIBITOR OF THE ENZYME P-HYDROXYPHENYLPYRUVATE DIOXYGENASE" FEBS LETTERS, vol. 318, no. 2, March 1993, pages 162-166, XP002028049 see the whole document ---	15
X	BARTA I C ET AL: "BENZOYLCYCLOHEXANEDIONE HERBICIDES ARE STRONG INHIBITORS OF PURIFIED P-HYDROXYPHENYLPYRUVIC ACID DIOXYGENASE OF MAIZE" PESTICIDE SCIENCE, vol. 45, no. 3, 1 November 1995, page 286/287 XP000547268 see the whole document ---	15
X	EP 0 614 970 A (HOECHST SCHERING AGREVO GMBH) 14 September 1994 see the whole document ---	15
P,X	WO 96 38567 A (RHONE POULENC AGROCHIMIE ;SAILLAND ALAIN (FR); ROLLAND ANNE (FR);) 5 December 1996 see sequence ID no. 2 ---	1,2
P,X	BARTLEY, G.E., ET AL.: "Arabidopsis thaliana p-hydroxyphenylpyruvate dioxygenase (HPD) mRNA, complete cds." EMBL SEQUENCE DATABASE, REL. 51, 19-MAR-1997, ACCESSION NO. U89267, XP002041908 see sequence ---	1,2,20
A	EP 0 652 286 A (RHONE POULENC AGROCHIMIE) 10 May 1995 see page 7, line 35 - line 47 ---	10,16,18
A	MISAWA N ET AL: "EXPRESSION OF AN ERWINA PHYTOENE DESATURASE GENE NOT ONLY CONFERS MULTIPLE RESISTANCE TO HERBICIDES INTERFERING WITH CAROTENOID BIOSYNTHESIS BUT ALSO ALTERS XANTHOPHYLL METABOLISM IN TRANSGENIC PLANTS" PLANT JOURNAL, vol. 6, no. 4, 1994, pages 481-489, XP002017203 see the whole document ---	10,16,18

-/--

4

INTERNATIONAL SEARCH REPORT

Intern: al Application No
PCT/US 97/11295

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DENOYA C D ET AL: "A STREPTOMYCES AVERMITILIS GENE ENCODING A 4-HYDROXYPHENYLPYRUVIC ACID DIOXYGENASE-LIKE PROTEIN THAT DIRECTS THE PRODUCTION OF HOMOGENTISIC ACID AND AN OCHRONOTIC PIGMENT IN ESCHERICHIA COLI" JOURNAL OF BACTERIOLOGY, vol. 176, no. 17, September 1994, pages 5312-5319, XP002028042 see the whole document ---	17
A	NORRIS, S.R., ET AL.: "Gnetic dissection of carotenoid synthesis in Arabidopsis defines plastoquinone as an essential component of phytoene desaturation" THE PLANT CELL, vol. 7, December 1995, pages 2139-2149, XP002041909 cited in the application see the whole document -----	1-20

4

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internal Application No
PCT/US 97/11295

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0614970 A	14-09-94	DE 4305696 A	01-09-94
		CA 2116421 A	26-08-94
		JP 6343464 A	20-12-94
WO 9638567 A	05-12-96	FR 2734840 A	06-12-96
		FR 2734841 A	06-12-96
		FR 2734842 A	06-12-96
		AU 6228696 A	18-12-96
EP 0652286 A	10-05-95	FR 2712302 A	19-05-95
		AU 7775194 A	18-05-95
		BG 99169 A	28-07-95
		BR 9404562 A	20-06-95
		CA 2135461 A	11-05-95
		CN 1121958 A	08-05-96
		CZ 9402743 A	13-09-95
		HU 70464 A	30-10-95
		JP 7184664 A	25-07-95
		NZ 264879 A	28-10-96
		PL 305775 A	15-05-95
		SK 134094 A	07-06-95
		US 5635618 A	03-06-97
		ZA 9408826 A	17-07-95